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Functional studies of elongation factor Tu from *Escherichia coli* : Site-directed mutagenesis and antibiotic actio

Krab, I.M.

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Functional studies of elongation factor Tu from *Escherichia coli*

Site-directed mutagenesis and antibiotic action

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Ivo Maarten Krab

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Co-promotor:	Dr. A. Parmeggiani
Referent:	Dr. B. Kraal
Overige leden:	Prof. Dr. J.-P. Abrahams Prof. Dr. J.F. Bol Prof. Dr. J. Reedijk

Voor mijn ouders

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Abbreviations

3D	three-dimensional
aa	amino acid
BSA	bovine serum albumin
cryo-EM	cryoelectron microscopy
DTT	dithiothreitol
EF	elongation factor
GAP	GTPase-activating protein
GBP	guanine nucleotide-binding protein
GEF	guanine nucleotide exchange factor
GMPPNP	guanosine 5'-[β , γ -imido]triphosphate
GNP, XNP	guanosine, resp. xanthosine di- or triphosphate
GST	glutathione <i>S</i> -transferase
IPTG	isopropyl- β -D-thiogalactopyranoside
ME	2-mercaptoethanol
MK	myokinase (EC 2.7.4.3)
PDB	the Brookhaven Protein Database of protein structures (www.pdb.org)
PEP	phospho <i>enol</i> pyruvate
PheRS	phenylalanine-tRNA synthetase (EC 6.1.1.20)
P _i	inorganic phosphate
PK	pyruvate kinase (EC 2.7.1.40)
PMSF	phenylmethylsulfonyl fluoride
TCA	trichloroacetic acid
wt	wild type

Chapter 1

General introduction

Protein biosynthesis is a remarkably intricate process, considering that it is based on the following simple principle: When a protein is needed in the cell, its genetic “recipe” stored in DNA is first copied into an intermediary transcript, messenger RNA (mRNA) (step 1: *transcription*), which is subsequently used as a template to build the protein upon by the molecular machine called ribosome and its multitude of help factors (step 2: *translation*). This principle has been called the “Central Dogma” of (molecular) biology (Crick, 1958).

The special properties of proteins that make them such versatile tools for living organisms derive from their structure: the specific sequence of amino acid residues in the chain makes each protein “fold up” into a three-dimensional shape characteristic for that protein and entirely determined by the order of the residues. This orients some of its amino acid residues in space in the right way to specifically bind other molecules or catalyze chemical reactions (enzymes). Often, studying the structure of a protein allows one to make deductions about the function of a protein. However, proteins are by no means static, rigid entities. They are flexible, and their ability to change shape is often an important part of their function.

It is generally assumed that early in evolution the major tools for life were not proteins, but RNA. Indeed, still today the machinery that produces proteins heavily relies on RNA as the functional component. The ribosome, whose structure has very recently finally become known in minute detail, consists of large RNA molecules with associated proteins. However, the RNA is not just there as a “scaffold” to hold together the proteins which would do the work, as has been thought in the past. It seems to be more the other way around, with the proteins lending support to the RNA, to increase its stability and efficiency.

Whether or not it was RNA by itself—in the form of the ribosomal RNA—that first started to string together amino acids into proteins, the fact is that the modern incarnation of the process depends for its speed and accuracy on the participation of proteins. For example, the high-speed delivery and selection of the amino acid to be built in during the production (elongation) phase of the process is taken care of by a protein called *Elongation Factor Tu* (*EF-Tu*). Since the realization of its role in the mid-sixties it has been the focus of many biochemical studies, serving in many ways as a model system. The latter is especially true since it was discovered that an impressive number of proteins in the eukaryotic cell involved in information exchange and transport (the G-protein superfamily) have their basic structure and functional aspects in common with the major domain of EF-Tu. Despite the understanding gained, the functioning of EF-Tu remains elusive on several points, for example concerning its enzymatic activity, and the way it can change its shape (conformation) in a most dramatic way.

EF-Tu binds a $\text{GTP}\cdot\text{Mg}^{2+}$ complex, which induces in the protein a conformation that in its turn has a high affinity for aminoacylated (aa)-tRNA, the activated amino acid bound to an adapter RNA molecule that is the actual building block for proteins. This complex of EF-Tu, $\text{GTP}\cdot\text{Mg}^{2+}$ and aa-tRNA, called the ternary complex, drifts around until it encounters a ribosome that is translating messenger RNA. Messenger RNA contains a sequence of “three-

letter words” called codons, each of which stands for a specific amino acid to be added to the protein chain. Each aa-tRNA has an anticodon that is shape-complementary to the codon that designates its amino acid. If the anticodon of the aa-tRNA in a ternary complex does not fit the current codon of the messenger on a translating ribosome, it is rejected. But when the aa-tRNA in a ternary complex “reads” its own codon in the receptor site (A site) on a ribosome (cognate codon-anticodon interaction), the ribosome triggers hydrolysis by EF-Tu of the bound GTP to GDP (the GTPase reaction). While EF-Tu can catalyze GTP hydrolysis in the absence of this interaction, the reaction triggered when a correct codon is read takes place in only milliseconds, several orders of magnitude faster than the intrinsic rate. Thus by some as yet unknown mechanism the cognate interaction of the ternary complex with the ribosome turns EF-Tu from a bad GTPase enzyme into a rather good one. As a result of the relatively small modification of its bound GTP to GDP, EF-Tu undergoes a surprisingly large change of shape, causing it to dissociate from the aa-tRNA which stays behind on the ribosomal A site. An exchange factor, EF-Ts, then intervenes to release the tightly bound GDP from the EF-Tu so that EF-Tu can rebind a GTP and start another cycle. The ribosome, after EF-Tu has left, adds the delivered amino acid to the growing protein chain and is prepared for the next round by another factor, EF-G, which shifts the mRNA along to the next codon of the message.

It was mentioned above that the function of a protein derives from its structure. For EF-Tu the structure of the four main states (GDP- and GTP-bound conformations and the complexes with aa-tRNA and EF-Ts) have been resolved by X-ray crystallography. However, the fact that EF-Tu changes conformation considerably between these states means that we need complementary information to understand the dynamic aspects of its function that are not revealed by the static structures. One technique that can provide this, is to modify by site-directed mutagenesis specific residues in the protein chain that we suspect to be important for its function, and characterize how this influences its properties.

When the work described in this thesis was started, there were some indications that EF-Tu amino acid residues involved in the binding of the Mg^{2+} •GTP complex, like Thr-25 and Asp-80, play a role in the GTPase reaction: mutations in these positions had been found to increase the intrinsic reaction (Harmark *et al.* (1992) and unpublished observations). A third residue that interacts with the magnesium ion, Thr-61, had been suggested to be involved in transmitting the ‘trigger’ signal from the ribosome (Ahmadian *et al.*, 1995). Residue His-84 had been proposed to have a direct catalytic action (Swart, 1987), although its mutation in a fragment of EF-Tu did not prevent catalysis (Cool & Parmeggiani, 1991). The crystal structure then revealed that His-84 was barred from access to the nucleotide by two conserved hydrophobic residues, Ile-60 and Val20. This led to the hypothesis that this barrier kept the intrinsic GTPase low, and conversely, that removing this barrier would increase the GTPase (Berchtold *et al.*, 1993). This thesis presents mutagenesis studies of the mentioned residues, aiming to clarify their role

in GTPase and the functioning of EF-Tu in general. Furthermore, it studies the relationship between nucleotide binding affinity of EF-Tu and its solubility in the cell on overexpression, a measure of its ability to fold correctly, and finally presents the characterization of the action of a new antibiotic that has EF-Tu as its target.

The structure of this thesis is as follows. First, *Chapter 2* summarizes the state of knowledge about EF-Tu and other relevant elements of the protein synthesis machinery in two parts. *Chapter 2a* was published as a review paper in 1998 and discusses more than 30 years of research on EF-Tu. *Chapter 2b* presents more recent developments that contribute significantly to our understanding of the translation process.

Chapters 3-5 describe the mutagenesis studies. *Chapter 3* focuses on residue Thr-25, a residue just following the loop that embraces the GTP/GDP nucleotide phosphates and has a direct interaction with the Mg^{2+} . While confirming its structurally central role in the nucleotide binding, the results also include several findings not readily predictable from the known 3D structures. *Chapter 4* examines mutants of Thr-61, Asp-80 and Ile-60, with results that are in various ways different from those of previous research and contradict the idea of a “hydrophobic barrier” effect. *Chapter 5* describes the properties of two mutations of residue His-84, showing that it is unlikely to play a specifically catalytic role in the intrinsic GTPase, although for the reaction with the ribosome it is essential. With these mutations we have now a fairly complete picture of the role of the various residues in the vicinity of the γ -phosphate.

Chapter 6 focuses on an *in vivo* functional question: the relationship between nucleotide binding and solubility in the cytoplasm of overexpressed EF-Tu variants. Starting point was the observation that on overexpression only a fraction of EF-Tu[D138N], which only binds xanthosine nucleotides that are not available in the cytoplasm, is soluble in the cytoplasm. This amount is similar to the concentration of EF-Ts of the cell, which prompted us to test if simultaneous overexpression of EF-Ts could improve the amount of soluble product. For this mutant and others that show a deficiency in nucleotide binding, a dramatic improvement was indeed observed, indicating that EF-Ts acts as a steric chaperon for the folding of these mutants. Moreover, this system also allowed to overcome so-called dominant negative effects of two mutations, T25A and D80N, on the growth of *E. coli*.

Chapter 7 describes the characterization of the action of a new antibiotic, enacyloxin IIa that acts on EF-Tu, affecting its intrinsic properties. This antibiotic represents the first member of a fourth structural class of EF-Tu-targeting agents. A comparison with the properties of the long-known antibiotic kirromycin reveals some similarities in the influence these two antibiotics exert on the factor, but also some striking differences.

Finally, in *Chapter 8* the findings presented in this thesis are discussed and summarized.

Chapter 2a

EF-Tu, a GTPase odyssey

A review of the literature

Ivo M. Krab and Andrea Parmeggiani

Biochimica et Biophysica Acta **1443**, 1-22 (1998)

- 1. Introduction**
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- 3. EF-Tu in the cell**
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 - 6.1 *The domains*
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1. Introduction

This review considers basic aspects of elongation factor (EF) Tu three decades after its discovery, with specific emphasis on selected open questions. EF-Tu was first isolated in 1964 in complex with EF-Ts, the so-called factor T (= transfer) that was separated into a temperature unstable (Tu) and a stable (Ts) component (for references: Lucas-Lenard & Lipmann, 1971). EF-Tu•GTP was identified as carrier of aa-tRNA to the A-site of mRNA-programmed ribosome, a function associated with hydrolysis of the bound GTP. The action of EF-Tu depended on whether GTP or GDP was bound, thus attributing to GTP hydrolysis a regulatory function (Kaziro, 1978). The many publications on EF-Tu in the 70s testify the great interest for this guanine nucleotide binding protein (GBP), a decade before this class of proteins was recognized as ‘on’ and ‘off’ switcher in a myriad of signalling processes in any organism (for references: Bourne *et al.*, 1990; Bourne *et al.*, 1991). Since the functional similarities of GBPs extend to their regulatory mechanisms, EF-Tu has been a useful model and an inexhaustible source of stimulating developments concerning function, structure and genetics.

As for all GTPases, EF-Tu•GTP is the ‘on’-state allowing the interaction with the targets aa-tRNA and ribosome, while EF-Tu•GDP is the ‘off’-state. In the traditional scheme of elongation the tight complex EF-Tu•GTP•aa-tRNA interacts with the mRNA-programmed ribosome carrying peptidyl-tRNA in the P-site. Codon-anticodon interaction triggers the hydrolysis of the bound GTP. Due to low affinity for aa-tRNA and the ribosome, EF-Tu•GDP is released, allowing the positioning of aa-tRNA in the ribosomal A-site and formation of a peptide bond between the NH₂ group of aa-tRNA and the C-terminal ester group of peptidyl-tRNA situated in the P-site. The translocation of peptidyl-tRNA from A- to P-site, promoted by the EF-G-dependent GTPase, displaces the discharged tRNA from P- to E-site, inducing a vacant A-site which allows a new elongation cycle to begin. Since EF-Tu has a higher affinity for GDP than for GTP, an efficient regeneration of the active form of EF-Tu needs both the specific GDP/GTP exchange factor (GEF) EF-Ts, that enhances kinetically the nucleotide

Table 1
Available 3D models of EF-Tu

EF-Tu complexes	PDB code
EF-Tu _{Ec} •GDP (2.5 Å) lacking the effector loop (Kjeldgaard & Nyborg, 1992)	
EF-Tu _{Tt} •GMPPNP (1.7 Å) (Berchtold <i>et al.</i> , 1993)	1EXM
EF-Tu _{Ta} •GMPPNP (2.5 Å) (Kjeldgaard <i>et al.</i> , 1993)	1EFT
EF-Tu _{Ta} •GMPPNP•aa-tRNA (2.7 Å) (Nissen <i>et al.</i> , 1995)	1TTT
EF-Tu _{Ec} •GDP (3.8 Å) with intact effector loop (Polekhina <i>et al.</i> , 1996)	
EF-Tu _{Ec} •GDP (2.5 Å) with intact effector loop (Abel <i>et al.</i> , 1996)	1DG1
EF-Tu _{Ta} •GDP (2.7 Å) with intact effector loop (Polekhina <i>et al.</i> , 1996)	1TUI
EF-Tu _{Ec} •EF-Ts _{Ec} (2.5 Å) (Kawashima <i>et al.</i> , 1996)	1EFU
EF-Tu _{Tt} •EF-Ts _{Tt} (3.0 Å) (Wang <i>et al.</i> , 1997)	1AIP

exchange on EF-Tu, and the interaction with aa-tRNA which increases the affinity for GTP thus forming a stable ternary complex. Several reviews have dealt with general (Lucas-Lenard & Lipmann, 1971; Miller & Weissbach, 1977; Bosch *et al.*, 1983; Weijland *et al.*, 1992; Kraal *et al.*, 1993) and specific (Parmeggiani & Swart, 1985; Abel & Jurnak, 1996; Clark & Nyborg, 1997) aspects of EF-Tu.

2. 3D Structure

EF-Tu from bacteria (from *Escherichia coli*, *Ec*, if not otherwise stated by subscripts: *Thermus thermophilus*, *Tt*, and *T. aquaticus*, *Ta*; *Salmonella typhimurium*, *St*) and its eukaryotic/archeal counterpart (EF-1 α) is a monomeric protein with molecular weight of 40-52 kDa. The first successful attempts to crystallize EF-Tu in complex with EF-Ts or GDP go back to 1968-71 (Parmeggiani, 1968; Miller & Weissbach, 1970; Arai *et al.*, 1972), X-ray grade crystals being obtained in 1973 (Snedden *et al.*, 1973). The breakthrough came in 1985 when the 3D structure of the nucleotide

binding domain from trypsinized EF-Tu•GDP lacking effector region residues 44-58 was resolved (Jurnak, 1985; La Cour *et al.*, 1985) and later refined to include all 3 domains (Kjeldgaard & Nyborg, 1992). Today 3D models exist for both states of EF-Tu, for EF-Tu•EF-Ts and EF-Tu•GTP•aa-tRNA (Table

Table 2
Specific regions of *E. coli* domain 1

Consensus motifs ^a	I (G ₁₈ HVDHGKT ₂₅); II (D ₈₀ CPG ₈₃); III (N ₁₃₅ KCD ₁₃₈); S ₁₇₃ AL ₁₇₅
P-loop	G ₁₈ HVDHG ₂₃
Effector region	G ₄₁ -T ₆₅
Switch regions	1 (D ₅₁ -T ₆₅); 2 (G ₈₃ -K ₉₀)

^aFor nucleotide binding. The last, SAL motif is conserved for prokaryotic elongation factors, but shows variations between GBP families.

1). Fig. 1 illustrates those of EF-Tu•GTP (A) and EF-Tu•GDP (B), its complexes with EF-Ts (C) and aa-tRNA (D) with for comparison that of EF-G•GDP (E).

The trypsinized EF-Tu•GDP showed a surprising hole between the 3 domains, which was no artefact, as proved by the 3D model of intact EF-Tu•GDP_{Ec/Ta} (Abel *et al.*, 1996; Polekhina *et al.*, 1996). EF-Tu in complex with GTP analogs has been elucidated at high resolution only

from thermophilic species (*Tt* HB8 and *Ta*). It is more compact, without hole and with extensive contacts between the 3 domains (Berchtold *et al.*, 1993;

Table 3
Topology of *E. coli* domain 1

β 1-L1[motif I]- α 1-L2[α 1'- α 1'' (GTP form)]/ α 1'- β -hairpin (GDP form)]- β 2-L3- β 3-L4[motif II]- α 2-L5- β 4-L6- α 3-L8- β 5[motif III]-L9- α 4-L10- β 6[SAL motif]-L11- α 5-L12- α 6-L bridge to domain 2
--

Kjeldgaard *et al.*, 1993). Domain 1 (aa: 1-199) has a 6 β -strand core connected by loops and α -helices in a fold shared by all GBPs even with low (<20%) primary sequence identity. The nucleotide binding pocket is delimited by the GBP consensus motifs (Kjeldgaard *et al.*, 1996), to the definition of which EF-Tu contributed prior to the availability of any 3D structures

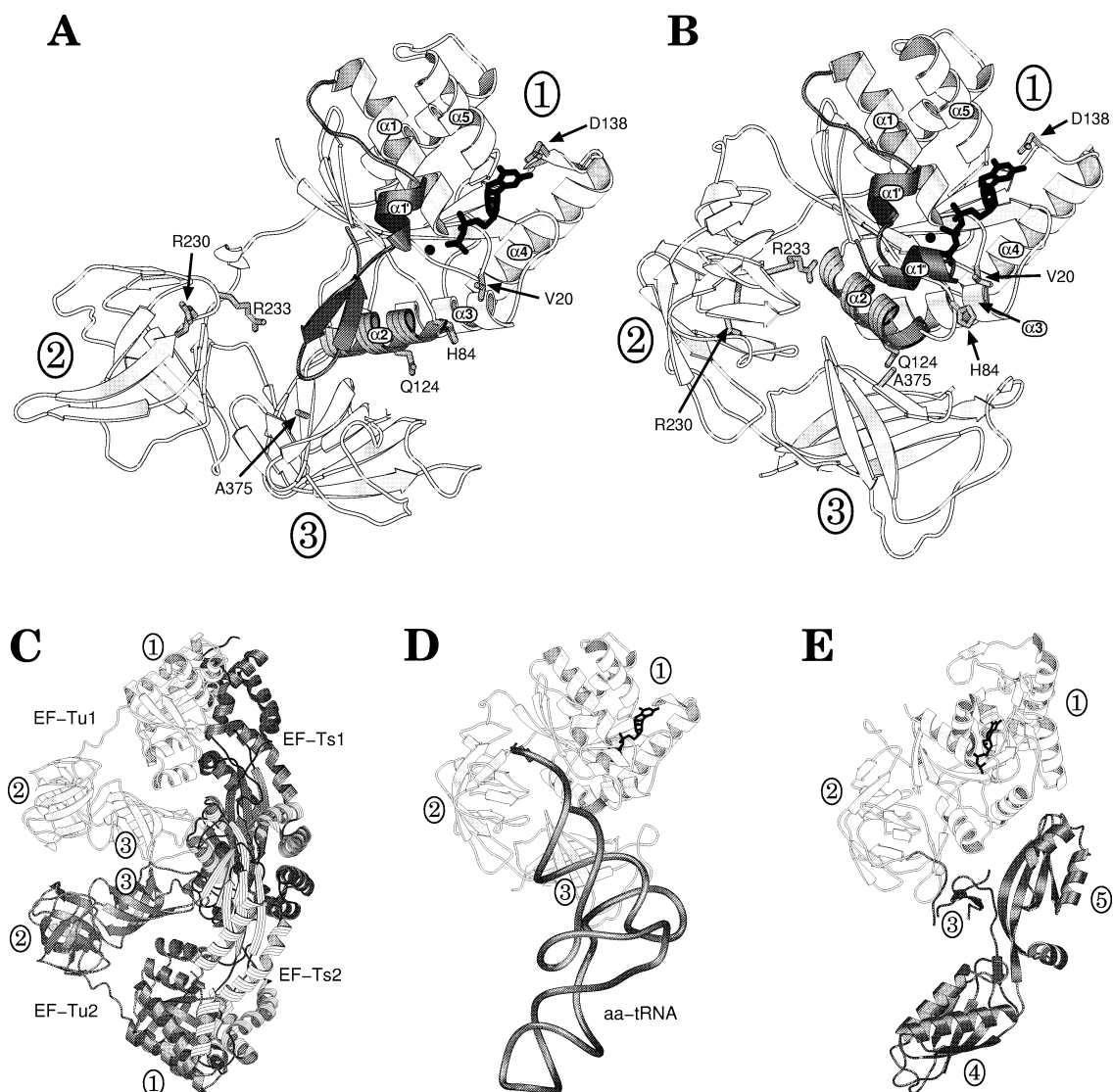


Figure 1 — 3D structure of EF-Tu in the GDP and GTP form, EF-Tu•EF-Ts, EF-Tu•GTP•aa-tRNA and EF-G•GDP

EF-Tu_{Td}•GDP (**A**) (PDB accession code 1TUI) and EF-Tu_{Tt}•GMPPNP (**B**) (coordinates provided by Dr. R. Hilgenfeld), showing the location of some mentioned residues (numbering of *E. coli*) and α -helices; the tetramer [EF-Tu_{Ec}•EF-Ts_{Ec}]₂ (**C**) [1EFU], the ternary complex EF-Tu_{Td}•GMPPNP•Phe-tRNA^{Phe} (**D**) [1TTT] and EF-G_{Tt}•GDP (**E**) [1DAR]. In panels **A** and **B**, big numbers designate the domains; the effector region is highlighted in dark and α -helix 2 is striped. Note the presence of one α -helix and a β -hairpin in the effector region in **A**, and two α -helices in **B** and the difference in orientation of helix 2. Also in panels **C**, **D** and **E** the encircled numbers indicate the various domains. In panels **D** and **E**, aa-tRNA and the corresponding domains in EF-G•GDP (3,4,5) are dark. In Figure **E** the domain 2 has the same orientation as domain 2 in **D**. Note however the quite different orientation of the domains 1. Drawing was made using SETOR and Setorplot (Evans, 1993)

(Tables 2 and 3). Domains 2 (aa: 209-299) and 3 (aa: 300-393) contain only β -strands and together behave as a rigid unit in the large conformational change from GDP- to GTP-bound state, which leads to a displacement of certain residues by as much as ~ 40 Å (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993). This transition is the most dramatic shown for any GBP. The structure elements most affected are the so-called switch regions. Switch 1 in the effector region comprises two short α -helices (1' and 1''), of which 1'' unwinds to an extended β -hairpin structure in the GDP state, forming a bridge to domain 3. Switch 2 comprises loop L4 and α -helix 2, which from the GDP to the GTP-state reorients through a 42° angle, shifting by four residues along the polypeptide chain towards the C-terminus. The resulting displacement of G83 permits the acceptance of the γ -phosphate.

The bound nucleotide is coordinated with a Mg^{2+} which in turn is coordinated to hydroxyl groups of conserved residues from motif I and the effector region (named after the homologous p21 region interacting with effectors), and with water molecules which hydrogen-bond to conserved aspartates in motif II and effector region.

EF-Tu•EF-Ts is the first GBP•GEF complex elucidated. In the crystal, two EF-Tu_{Ec}•EF-Ts_{Ec} form a heterotetramer in which the two EF-Ts interact extensively and each EF-Ts interacts with one EF-Tu (Kawashima *et al.*, 1996). The major contacts between EF-Tu and EF-Ts take place on domain 1 and involve loop L2, the N-terminus of helix 2 — tightly associated with domain 3 — and α -helices 3 and 4. Besides domain 1, only the tip of domain 3 interacts directly with EF-Ts. Noteworthy, EF-Ts F81 (sF81) intrudes between α -helices 2 and 3 (see Section 6.4). The conformation of EF-Ts in the complex is close to that of EF-Tu•GDP. The quaternary structure of EF-Tu_{Tt}•EF-Ts_{Tt} is different and consists of a diad symmetric heterotetramer in which each EF-Ts interacts with both EF-Tu (Wang *et al.*, 1997). The basic traits of the interaction surface are otherwise similar to that of the *E. coli* complex.

Phe-tRNA binds to EF-Tu•GMPPNP in the interface of domains 1/2 and 1/3 (Nissen *et al.*, 1995), the anticodon pointing away from EF-Tu. There are three major areas of contact: domain 3 with one side of the T-stem, the three-domain crosspoint with the 5' phosphate, and the cleft between domains 1 and 2 accommodating the tRNA-bound amino acid. The conformation of EF-Tu•GTP remains essentially the same upon interaction with aa-tRNA. Remarkably, the ternary complex and EF-G•GDP share a similar overall shape (see Section 7).

Though EF-Tu and EF-1 α fulfill the same function in elongation, there are some notable differences in their primary structure. Whereas in EF-Tu's the last amino acid of the first nucleotide-binding consensus element is a Thr, in EF-1 α it is a Ser, like in most small GBPs. An important functional region with sequence divergence between the two main branches of the universal tree is the hypervariable part of the effector region, that in EF-1 α is 15 aa longer than the average EF-Tu and contains many charged residues (four to five Glu and six Lys/Arg). Since this region is thought to be important for interaction with the ribosome, this may be a factor in the inability of EF-Tu and EF-1 α to substitute for each other (Slobin, 1981). The

specialized *E. coli* EF SelB, that is responsible for the incorporation of selenocysteine into the polypeptide chain (Baron & Böck, 1995), shows a very short effector region and some structural differences in the guanine binding (Hilgenfeld *et al.*, 1996).

3. EF-Tu in the cell

EF-Tu is ubiquitous in all kingdoms, therefore it has proved very useful to define evolutionary relationships between all organisms and locate the root of the universal tree (Baldauf *et al.*, 1996). It is the most abundant bacterial protein (5-10% of the total proteins), its concentration (0.1-0.2 mM) being equivalent to that of aa-tRNA and ~10-fold higher than that of ribosomes (for references: Bosch *et al.*, 1983). Nearly all cellular aa-tRNA is trapped by EF-Tu•GTP (Gouy & Grantham, 1980).

3.1 Genetics

Exceptionally for a bacterial protein, EF-Tu_{Ec} is encoded by two unlinked genes: *tufA* at 72 min in the linkage map in the *str* region and *tufB* at 88 min in the *rif* region, in operons containing genes of other components of the protein synthesizing machinery (for references: Bosch *et al.*, 1983). The sequences of *tufA* and *tufB* are identical but for 13 bases, primarily in the wobble position of the codon; in fact, only the C-termini of the products are different: Gly in EF-TuA and Ser in EF-TuB, which does not cause any observable functional difference (Anborgh *et al.*, 1991). *Tuf* gene duplication occurs in other bacteria than *E. coli*, particularly Gram-negative, whereas Gram-positive bacteria have often one gene, except some clostridia and streptomyces (for references: Vijgenboom *et al.*, 1994). In *S. typhimurium* the two genes are closely linked by gene conversion events (Abdulkarim & Hughes, 1996). Consequently, the genes are 99% identical at the nucleotide level and 100% at the aa level, differing from EF-TuA_{Ec} only in residue 189. Either *tuf* gene can be inactivated without loss of viability (Hughes, 1990) reducing the growth rate by only ~30 % (Tubulekas & Hughes, 1993a). Also in *E. coli* either gene is dispensable (A. Zuurmond and B. Kraal, personal communication). *E. coli* *tufA* expression seems not to be under growth rate control, while that of *tufB* is under growth rate and stringent control (for references: Lindahl & Zengel, 1986) and regulated by a transactivation element identified as FIS (factor of inversion stimulation) (Verbeek *et al.*, 1992). The control of *tufA* expression and the coordinated regulation of the two *tuf* genes represent important unclarified aspects. Because of the stoichiometric role of EF-Tu in protein biosynthesis this redundancy may have evolutionary reasons, guaranteeing to the organism a better survival in hostile conditions. In streptomyces species including EF-Tu-specific antibiotic producers one finds different situations. Apart from the major gene (*tuf1*) coding for the constitutively expressed elongation factor, one or two other *tuf*-like genes are present, which in contrast to *E. coli* or *S. typhimurium* differ considerably from *tuf1*. *S. ramocissimus* *tuf2* and *tuf3* show 89 and 63 % identity to *tuf1*, respectively (Vijgenboom *et al.*, 1994; Kraal *et al.*,

1995). *S. collinus*, *S. lividans* and *S. coelicolor* have besides *tuf1* only the equivalent of *tuf3* (van Wezel *et al.*, 1994; Vijgenboom *et al.*, 1994). These genes are normally not expressed, but *tuf3* of *S. coelicolor* is subject to positive stringent control (van Wezel *et al.*, 1995). Whether the products of *tuf2* and 3 are functional in elongation *in vivo* remains to be determined. Some actinomycetes other than streptomycetes were found to have only one *tuf* (van Wezel, 1994) but *Planobispora rosea*, the producer of the EF-Tu-specific antibiotic GE2270 A has two genes (*tuf1* and *tuf3*) (Möhrle *et al.*, 1997).

3.2 Posttranslational modifications

EF-Tu is N-terminally acetylated and the nonconserved lysine 56 can be mono- or dimethylated (Arai *et al.*, 1980; Jones *et al.*, 1980). Phosphorylation *in vivo* of the conserved T382, situated in the interface 1/3 that is involved in kirromycin resistance, has been reported (Lippmann *et al.*, 1993) on a minor fraction of EF-Tu (<10%). *In vitro*, EF-Tu can be somewhat phosphorylated by a ribosome-associated kinase (Alexander *et al.*, 1995). The extent of phosphorylation is enhanced by EF-Ts and phosphorylated EF-Tu does not interact with kirromycin or aa-tRNAs. The physiological significance of this modification, proposed to have a regulatory role, awaits further clarification.

3.3 Translation, speed and accuracy

In the exponentially growing cell, the amino acid incorporation rate is of 10-20 aa s⁻¹ (for references: Bremer & Dennis, 1987), each elongation cycle lasting 50-100 ms. Since simple codon-anticodon interaction is not sufficient to ensure the 10⁻³-10⁻⁴ overall missense error frequency as found *in vivo* (for references: Bouadloun *et al.*, 1983), a model for translational fidelity was proposed (Hopfield, 1974; Ninio, 1975) and since then analyzed by a wealth of *in vivo* and *in vitro* studies (for references: Dix *et al.*, 1990; Kurland *et al.*, 1990). The model consists of two main steps: the initial selection of the proper ternary complex by the mRNA-programmed ribosome, rejecting noncognate ternary complexes (a step analyzed in fast kinetic experiments using fluorescent substrates (Rodnina *et al.*, 1995; Rodnina *et al.*, 1996)), and a proofreading process following GTP hydrolysis, discarding near-cognate ternary complexes. Therefore, at each step aa-tRNA can either proceed along the path toward peptide bond formation or leave the ribosome. In proofreading, finer differences, such as those between near-cognate anticodons, can be discriminated on the basis of their affinity to the mRNA. The influence of the EF-Tu concentration on the dynamics and accuracy of protein synthesis has also been evaluated by computed models (Pingoud *et al.*, 1990).

Mutants of EF-Tu, tRNA and ribosomes can affect the fidelity of translation (Tapio & Kurland, 1986; Tapio & Isaksson, 1990). In general, mutant EF-Tu with slower GTPase and protein biosynthesis (Swart *et al.*, 1987; Jacquet & Parmeggiani, 1989; Zeidler *et al.*, 1995) are hyperaccurate, due to a longer pausing on the ribosome. The degree of fidelity in translation

represents an optimized compromise between time and energy consumption (as GTP hydrolysis) on one side and the required accuracy on the other side (for references: Dix *et al.*, 1990; Kurland *et al.*, 1990). Interestingly, in vivo combination of EF-TuA[A375T] and EF-TuB[G222D] causes nonsense read-through, while either mutant in combination with wild-type does not (Vijgenboom *et al.*, 1985). Although it was reported that EF-TuA[A375T] by itself is error-prone in vitro (Tapio & Kurland, 1986), cooperative phenomena between mutant EF-Tu factors were also found in translational +1/-1 frameshifts in vivo (Hughes *et al.*, 1987; Vijgenboom & Bosch, 1989). These findings raise the question whether cooperative effects can also take place between two wt EF-Tu during translation of sense codons (see Section 9).

3.4. A multifunctional role

The abundance of EF-Tu in the cell has inspired research on possible functions other than in elongation. For a proposed actin-like structural role (Jacobson & Rosenbusch, 1976) no evidence was found by immunoelectronmicroscopy (Schilstra *et al.*, 1984). Polymerization in vitro was observed, but only in unphysiological conditions (Beck *et al.*, 1978; Leberman, 1984; Helms & Jameson, 1995). Other observations remained without follow-up: enhancer of rRNA synthesis, an effect inhibited by ppGpp (Travers, 1973); association with the periplasmic space and outer membrane (Dombou *et al.*, 1981; Sedgwick & Bragg, 1986; Young & Bernlohr, 1991). Unquestioned is its role as one of the four subunits of the replicase of RNA phages, where EF-Tu acts in complex with EF-Ts in the initiation step (Blumenthal *et al.*, 1972; Landers *et al.*, 1974). A recent novelty is the chaperon-like activity (see Section 10). One should mention that interaction with the cytoskeleton was found for EF-1 α as part of actin- and tubulin-based polymers (for references: Condeelis, 1995).

4. EF-Tu in vitro

The in vitro properties of purified EF-Tu have been the object of many detailed studies. Remarkably, its affinity for GDP (in the nM range) is two orders of magnitudes higher than for GTP, mainly due to a low dissociation rate (Fasano *et al.*, 1978). No reason for this differential affinity in terms of 3D structure has as yet been reported. In EF-Tu_{Tt} the difference in affinity is smaller (Wagner *et al.*, 1995) and it is absent in EF-1 α (Cr chet & Parmeggiani, 1986a). Chelation of Mg²⁺ by EDTA reduces by 10³ the affinity for GDP (Miller & Weissbach, 1977), providing a convenient way to remove the nucleotide (Fasano *et al.*, 1982a). Nucleotide-free EF-Tu loses its activity (~10% hr⁻¹) but within a few hours can be reactivated by GDP, particularly in combination with the antibiotic kirromycin (Fasano *et al.*, 1982a), while traces of GDP (< 5%) conserve the activity of nucleotide-free EF-Tu for several days and association with EF-Ts for months (Parmeggiani & Sander, 1981). On the more stable EF-Tu_{Tr}, bound GDP is removed by 5 M urea without irreversible denaturation (Seidler *et al.*, 1987). EF-Ts enhances the dissociation of EF-Tu•GDP somewhat better than of EF-Tu•GTP

(~6-fold) (Fasano *et al.*, 1978), in line with the observation that the EF-Tu 3D structure in EF-Tu•EF-Ts is similar to that of EF-Tu•GDP (Kawashima *et al.*, 1996). This difference, together with the sequestration of EF-Tu•GTP by aa-tRNA and the higher concentration of GTP in the cell (1 vs 0.1 mM GDP: Neuhaard & Nygaard, 1987), favors the physiologically relevant EF-Tu•GDP/GTP exchange in the cell. The stimulatory effect of EF-Ts on EF-Tu•GDP can reach values up to $7 \cdot 10^5$ -fold (Chau *et al.*, 1981), ie comparable to that of GEF on H-ras p21 (10^5 -fold: Lenzen *et al.*, 1998).

EF-Tu hydrolyzes GTP very poorly ($\sim 1 \text{ mol mol}^{-1} \text{ EF-Tu hr}^{-1}$), an intrinsic activity that can be enhanced by monovalent cations (Fasano *et al.*, 1982b), vacant ribosomes, aa-tRNA (Parlato *et al.*, 1983; Jacquet & Parmeggiani, 1988) and kirromycin (Wolf *et al.*, 1974), from 5 to 100-fold. The strongest enhancer by far is the mRNA-programmed ribosome with occupied P-site, that following cognate codon-anticodon interaction induces one-round GTP hydrolysis in the range of ms, 10^5 faster than the intrinsic GTPase (calculated from: Swart *et al.*, 1987; Bilgin *et al.*, 1992). Thus, ribosomes can be considered for EF-Tu the equivalent of GAP for ras proteins. In fast kinetic experiments the GTP hydrolysis rate is reduced by a factor of 10^4 when the binding ternary complex is non-cognate and the E-site of the programmed ribosome is empty (Rodnina *et al.*, 1995; Rodnina *et al.*, 1996). A similar effect is observed for the GTPase of EF-Tu in complex with nonacylated tRNA upon binding to programmed ribosomes, where a cognate tRNA stimulates, but a noncognate tRNA inhibits (Swart & Parmeggiani, 1989). Even a tRNA lacking CCA end is able to stimulate the GTPase in these conditions (Picone & Parmeggiani, 1983).

The possibility to reproduce in vitro in simple purified systems — most popular of which is poly(U)-directed poly(Phe) synthesis — a protein biosynthesis rate near to that in vivo (Wagner *et al.*, 1982; Bartetzko & Nierhaus, 1988), underlines the validity of analytic studies in vitro for understanding physiological mechanisms. Also translation of a natural messenger can be achieved in vitro with near in vivo elongation rate (Pavlov & Ehrenberg, 1996). However, early studies have identified other factors necessary to achieve optimal in vitro translation (for references: Ganoza *et al.*, 1996). This should be kept in mind when interpreting results obtained with minimal purified systems, such as stoichiometry questions (see Section 9).

5. EF-Tu and antibiotics

EF-Tu is specifically affected by four different kinds of antibiotics and to date represents after the ribosome the major target of inhibitory agents in protein biosynthesis (for references: Parmeggiani & Sander, 1980; Parmeggiani & Swart, 1985; Cetin *et al.*, 1998). Besides their interest as antimicrobial agents, antibiotics have been important for studying EF-Tu intrinsic activities, its interaction with ligands and the visualization of the EF-Tu•GTP binding site on the ribosome. The first identified and best studied is kirromycin (=mocimycin, for references: Parmeggiani & Sander, 1980; Parmeggiani & Swart, 1985), a prototype of a family comprising

at least 15 analogs. The others are pulvomycin, GE2270 A (= MDL 62,879: Anborgh & Parmeggiani, 1991) and enacyloxin IIa (Cetin *et al.*, 1996). These antibiotics have different chemical structures, but their actions share a number of similarities. They all strongly increase the affinity of EF-Tu•GTP, slowing down its dissociation rate, while they influence the interaction with GDP to various extents. Kirromycin markedly enhances the intrinsic GTPase activity of EF-Tu (up to 100-fold at high concentrations of monovalent cations: Ivell *et al.*, 1981). EF-Tu•GTP•kirromycin has a reduced affinity for aa-tRNA (Pingoud *et al.*, 1978; Abrahams *et al.*, 1991), while EF-Tu•GDP•kirromycin can form an anomalously stable ternary complex (Chinali *et al.*, 1977). Consequently, on the ribosome EF-Tu•GDP is not released after GTP hydrolysis, thus blocking this and all following ribosomes on the polysome, which explains the recessive character of kirromycin resistance. Pulvomycin and GE2270 A display similar mechanisms of action, different from that of kirromycin, and abolish the interaction with aa-tRNA, as if EF-Tu•GTP were frozen in a GDP-like ‘off’-state (Wolf *et al.*, 1978; Anborgh & Parmeggiani, 1993). Enacyloxin IIa, the most recently identified EF-Tu-specific antibiotic, inhibits the formation of a new peptide bond by blocking the C-terminal incorporation of the tRNA-bound amino acid into the polypeptide chain situated in P-site, like kirromycin (Cetin *et al.*, 1996), without, however, blocking the release of EF-Tu•GDP. Since enacyloxin IIa can also inhibit the incorporation of the tRNA-bound amino acid in the absence of EF-Tu, at least at high Mg^{2+} concentrations, it appears to be able to directly affect the A-site. No method has yet been found to remove pulvomycin or GE2270 A once bound to EF-Tu. In contrast, competition with EF-Ts for binding to EF-Tu allows the release of the tightly bound kirromycin (Chinali *et al.*, 1977). Differently from kirromycin, enacyloxin IIa only binds transiently to EF-Tu, but similarly, it competes with EF-Ts for binding to EF-Tu. For the EF-Tu binding sites of these antibiotics see Section 6.5.

Kirromycin allowed for the first time the selection of EF-Tu mutants. The presence of two *tuf* genes and the dominant character of the mechanism of action of this antibiotic require that both *tufs* are altered to express a resistant phenotype. Accordingly, the first two *E. coli* strains with resistant EF-Tu isolated contained either only EF-TuA[A375V] or in addition to EF-TuA[A375T] a mutated EF-TuB[G222D] (for references: Duisterwinkel *et al.*, 1984), displaying a recessive kirromycin sensitivity that resulted from a deficient interaction with the ribosome (Swart *et al.*, 1987). EF-TuB[G222D], though active in vitro at high Mg^{2+} concentrations, is likely inactive in the cell (Tapio & Kurland, 1986; Talens *et al.*, 1996). In *S. typhimurium* it was possible to identify a kirromycin-resistant EF-TuB (Tuohy *et al.*, 1990). In this organism, as exception to the recessivity of kirromycin resistance, kirromycin-resistant EF-TuB becomes dominant over a sensitive EF-TuA in the presence of ribosomes which are error restricted and streptomycin resistant (Tubulekas *et al.*, 1991). Some evidence in vivo and in vitro (Zeef *et al.*, 1994) has suggested that pulvomycin sensitivity may be dominant over resistance. These results should be reexamined, since they disagree with the action of this

antibiotic preventing the interaction between EF-Tu and aa-tRNA and thus the binding to the programmed ribosome. Moreover, resistance to GE2270 A, that shares with pulvomycin most functional properties, is not recessive (Möhrle *et al.*, 1997).

Eukaryotic protein biosynthesis is not affected by kirromycin, that nevertheless increases the rate of dissociation of the EF-1 α •nucleotide complex (Créchet & Parmeggiani, 1986b). The lack of an effect is likely due to overlapping binding sites of kirromycin and the ribosome.

It is probable that other as yet unknown antimicrobial agents exist that act specifically on EF-Tu. Their potential use for therapeutic applications encourages further research.

6. EF-Tu dissected

This section resumes what is known about the contribution of the various part of EF-Tu to its functions.

6.1 The domains

The isolated EF-Tu domain 1, named G domain (Parmeggiani *et al.*, 1987), binds GDP and GTP with near-equal μ molar affinity close to that of EF-Tu•GTP and sustains GTP hydrolysis. G-domain_{Ec/Tt} is virtually irresponsive to or only weakly interacts with the other ligands (Parmeggiani *et al.*, 1987; Nock *et al.*, 1995; Cetin *et al.*, 1998). Therefore, domains 2 and 3 are essential for the action of macromolecular ligands. EF-Tu lacking either domain 2 (EF-Tu[Δ 2]) or 3 (EF-Tu[Δ 3]) is inactive in poly(Phe) synthesis and has intrinsic GTPase activity and μ M affinities for GTP and GDP similar to G-domain (Cetin *et al.*, 1998). Thus, all three domains are needed for the differential affinity towards GTP and GDP, indicating that domains 2 or 3 can influence the nucleotide binding pocket by as yet unknown long-range effects. In contrast to EF-Tu_{Ec}[Δ 3], EF-Tu_{Tt}[Δ 3] was reported to sustain a much higher activity than the G domain_{Tt} and the intact EF-Tu_{Tt} (Nock *et al.*, 1995). EF-Tu[Δ 2] and EF-Tu[Δ 3] respond to EF-Ts and somewhat to aa-tRNA, but not to ribosomes (Cetin *et al.*, 1998). Concerning antibiotics, only EF-Tu[Δ 3] responds significantly. The retention of part of the functional properties of the intact molecule and the analysis by various methods (CD spectra, thermostability and model building) indicates that the interaction forces between domains 1 and 2, and domains 1 and 3 are sufficiently strong to avoid significant displacements. NMR studies (Lowry *et al.*, 1991) have shown that also G-domain has an organized structure in the nucleotide binding site. Apparently, the individual domains of EF-Tu and their combinations constitute remarkably stable entities.

6.2 Nucleotide binding pocket

NMR studies have revealed that G domain K24 has nearly the same strong proton and nitrogen shifts as the homologous K16 of p21 (Lowry *et al.*, 1991). Also amino acid substitutions in the nucleotide binding pocket have stressed the similarity with p21 (for references: Weijland *et*

al., 1992). Noteworthy, whereas replacement of G-domain D80($\rightarrow$ N) coordinated to Mg^{2+} via a water molecule markedly enhances the GTPase activity (Harmark *et al.*, 1992), the same substitution in the intact EF-Tu exerts an inhibitory effect (I. M. Krab, unpublished), revealing constraints by domains 2 and 3 on the intrinsic G domain GTPase. Replacement of D138($\rightarrow$ N) in motif III, which interacts with position 2 of the guanine ring, modifies the substrate specificity of EF-Tu, supporting the binding and hydrolysis of XTP instead of GTP (Hwang & Miller, 1987; Weijland & Parmeggiani, 1993). Also in this case, EF-Tu has served as a model protein. In fact, the corresponding mutation in p21 (Zhong *et al.*, 1995; Schmidt *et al.*, 1996), the ras-like proteins Rab (Rybin *et al.*, 1996) and Ran (Sweet & Gerace, 1996) also changes the substrate specificity.

6.3 aa-tRNA

Aminoacylation of tRNA is essential for the formation of a stable ternary complex, since it increases up to $\sim 10^5$ -fold the affinity of uncharged tRNA for EF-Tu•GTP (Pingoud *et al.*, 1982b). The affinity of aa-tRNAs to EF-Tu•GTP varies ~ 10 -fold, mainly depending on the nature of the esterified amino acid (Pingoud & Urbanke, 1980; Louie *et al.*, 1984; Abrahamson *et al.*, 1985). The acceptor stem contains determinants for the discrimination of elongator and initiator tRNA (Schulman *et al.*, 1974; Nissen *et al.*, 1996). For the minimal structural elements required for an efficient interaction with EF-Tu•GTP see (Rudinger *et al.*, 1994). Recently, K89 and N90 substitutions were found to decrease the affinity for aa-tRNA, as predicted from their binding to the 5'-end of aa-tRNA, (Wiborg *et al.*, 1996). The negative influence on aa-tRNA binding by substituting H118 is less clear in terms of 3D structure and is very probably indirect via long-range effects (Jonák *et al.*, 1994; Wiborg *et al.*, 1996). The attachment of the 3' acceptor-stem of aa-tRNA, involving primarily domain 1, justifies why deletion of this domain (Peter *et al.*, 1990; Pieper *et al.*, 1990) abolishes the interaction with aa-tRNA, while removal of either one of domains 2 and 3 allows some residual binding (Cetin *et al.*, 1998). The 3D model agrees in large part with older results suggesting that the protection of aa-tRNA by EF-Tu concerns the aa-end, aa-stem, T-stem and extra loop on one side of the tRNA molecule (Parlato *et al.*, 1981; Wikman *et al.*, 1982; Parlato *et al.*, 1983).

6.4 EF-Ts

The 3D model of EF-Tu•EF-Ts shows that EF-Ts mainly interacts with domain 1 and to a very limited extent with domain 3 (Kawashima *et al.*, 1996). As compared to intact EF-Tu, EF-Ts is half as active on EF-Tu[$\Delta 2$] and EF-Tu[$\Delta 3$] and nearly inactive on G-domain. Removal of either one of the two noncatalytic domains and even more the removal of both domains already enhances the intrinsic dissociation of G domain•GDP (Parmeggiani *et al.*, 1987; Cetin *et al.*, 1998). These results show that domains 2 and 3 induce specific constraints on domain 1, that are relieved by EF-Ts. The ability of kirromycin or enacyloxin IIa to inhibit the EF-Ts-induced

GDP release on EF-Tu[Δ2] and EF-Tu[Δ3], as it does on intact EF-Tu, supports the non-artefactual nature of the response to EF-Ts in these constructs. EF-Tu_{Tt}[Δ1] can interact with EF-Ts_{Tt} (Peter *et al.*, 1990), an effect likely dependent on the EF-Ts interaction with domain 3.

As for the pathway for the EF-Ts signal accelerating the release the nucleotide from EF-Tu, in both EF-Tu_{Ec}•EF-Ts_{Ec} (Kawashima *et al.*, 1996) and EF-Tu_{Tt}•EF-Ts_{Tt} (Wang *et al.*, 1997) it was shown that EF-Ts F81 (sF81) intrudes into EF-Tu between α-helix2 and α-helix3. This was proposed either to destabilize residues involved in the coordination of the nucleotide-bound Mg²⁺, favoring the release of the nucleotide, or to elicit interactions that cause a peptide flip in the P-loop which leads to a displacement of the β-phosphate by V20 and breaks the hydrogen-bonds between this phosphate and the P-loop. The latter phenomenon is considered secondary by Kawashima *et al.* (Kawashima *et al.*, 1996). The observation that mutation of sF81 and the near-located sD80 (Zhang *et al.*, 1996) or of EF-Tu H118 (Jonák *et al.*, 1998) only partially decreases the response to EF-Ts and that the EF-Ts N-terminal region around sC22 is essential for the transmission of the release signal to the guanine base (Hwang *et al.*, 1997) favors multiple pathways, in agreement with the suggestion of Kawashima *et al.* (Kawashima *et al.*, 1996) that EF-Ts induces a movement of α-helix4, destabilizing ribose and base of the nucleotide.

6.5 Binding sites for antibiotics

Competition studies have suggested different binding sites on EF-Tu for kirromycin and pulvomycin (Pingoud *et al.*, 1982a) or GE2270 A (Landini *et al.*, 1996) and overlapping binding sites for pulvomycin and GE2270 A (Anborgh & Parmeggiani, 1991). The localization of the binding site for kirromycin and pulvomycin has drawn most efforts so far, due to the identification of several residues whose substitution induces resistance to the two antibiotics. The aa inducing kirromycin-resistance are clustered on the interface of domains 1/3 of EF-Tu•GTP (Q124, G316, A375 (Mesters *et al.*, 1994b); L120, Q124, Y160, G316, Q329, A375, E378 (Abdulkarim *et al.*, 1994)). In EF-Tu•GDP, only Q124 lies on the much smaller 1/3 interface. Resistance varies considerably depending on the residue: substitutions Q124 and A375, buried in the interface, induce a stronger resistance than the peripheral G316 (Mesters *et al.*, 1994b). From the 3D model the various mutations stabilize the interface 1/3 in the GTP state with the notable exception of Q124K that lies near to K357 (a residue reported to cross-link the antibiotic: van Noort *et al.*, 1984) and induces the strongest resistance, suggesting a direct effect on the binding of the antibiotic (Mesters *et al.*, 1994b). Binding of kirromycin in this interface would influence the transitions between EF-Tu•GTP and EF-Tu•GDP in agreement with its ability to influence most EF-Tu reactions, perhaps by reorienting α-helices 2 and 3 participating in this interface. Though many resistance mutations are found in domain 3, the influence of kirromycin on G domain and EF-Tu[Δ3] indicates that the main interaction of the antibiotic is with domain 1. Moreover, the competition with EF-Ts suggests that elements of

the EF-Tu•EF-Ts contact area (L1(P-loop), L6- α 3, α 4), of which L6- α 3 are involved in this interface, are implicated.

The residues whose substitution induces pulvomycin resistance (R230, the double mutation R230/R233, R333 and T334; (Zeef *et al.*, 1994; Boon *et al.*, 1995)) are located on domain 2 and 3 in the three-domain junction, an area crucial for the binding of aa-tRNA. The single mutations induce a modest resistance, whereas the effect of the double mutation is marked. From the 3D model, they should destabilize the three-domain interaction, an effect contrary to that of mutations inducing kirromycin resistance. The location of the binding site of pulvomycin is uncertain and the resistance induced by some of these mutations probably indirect, even though it likely contains common elements with the binding region of aa-tRNA. Concerning GE2270 A, mutation V228A in *Bacillus subtilis* EF-Tu (= V226 in EF-Tu_{Ec}) induces resistance to a GE2270 A analog (Shimanaka *et al.*, 1995), while a GE2270 A-resistant *B. subtilis* EF-Tu has substitution G278A (G275 in EF-Tu_{Ec}) (Sosio *et al.*, 1996). From the similarity of its action, GE2270 A and pulvomycin, or enacyloxine IIa and kirromycin may have overlapping binding sites (Cetin *et al.*, 1998). However, until visualisation of the bound antibiotic is achieved, a precise location of these binding sites remains largely speculative. So far, no visualization of cocrystallized GE2270 A (Abel *et al.*, 1996) or kirromycin (Kristensen *et al.*, 1996) has been reported.

Two mechanisms for expression of kirromycin resistance have been proposed (Mesters *et al.*, 1994b). The first is based on a decreased binding affinity of mutant EF-Tu for the antibiotic (Fasano & Parmeggiani, 1981; Van der Meide *et al.*, 1981), related to a stabilization of the interface 1/3; the second, operative at high kirromycin concentrations is associated with a reduced affinity of EF-Tu•GDP for kirromycin after GTP hydrolysis. High resistance includes both mechanisms (Kraal *et al.*, 1995). Moreover and worth mentioning, several kirromycin-resistant EF-Tu have decreased affinity for aa-tRNA, which should also facilitate the release of EF-Tu from the ribosome (Abdulkarim *et al.*, 1996).

7. The interaction with the ribosome

Multiple interactions between EF-Tu and ribosomal proteins were suggested already in the seventies and in the eighties by functional and cross-linking experiments. The role in the GTPase activity of EF-Tu of ribosomal subunits depleted of an increasing number of proteins ('cores') indicated that the 50S ribosomal subunit is responsible for stimulating the GTPase activity of EF-Tu, whereas the 30S ribosomal exerts a modulatory effect (Sander *et al.*, 1975; Sander *et al.*, 1976). Noteworthy, conditions were found in which even 'cores' lacking proteins L7/L12 stimulate the EF-Tu GTPase. The most probable region of the 50S ribosome binding to EF-Tu implicates an area including the stalk formed by the (L7-L12)₂ dimers, the nearby proteins L10 and L11, and the α -sarcin loop from domain V of the 23 S rRNA (Noller, 1991). In the 16S RNA of the 30S subunit, the 530-loop is also protected by EF-Tu (Powers &

Noller, 1993). The proteins S4, S5 and S12 that, like EF-Tu, are involved in the accuracy of peptide chain elongation are located nearby (Mueller & Brimacombe, 1997). These elements have been proposed to be implicated in the modulation of the function of EF-Tu by 30S subunits (Powers & Noller, 1993; Powers & Noller, 1994). Concerning the localization of EF-Tu on the ribosome during the elongation cycle, considerable progress has recently been made by cryoelectronmicroscopy visualizing at 18 Å resolution the quaternary complex of EF-Tu•kirromycin•GDP•aa-tRNA blocked on the ribosome (Stark *et al.*, 1997). Domain 1 is in contact with the C-terminal region of the (L7-L12)₂ stalk and the underneath region of the 50S subunit, and domain 2 is close to the 30S subunit on the side opposite to the platform where proteins S4, S5 and S12 are located. Worth mentioning, EF-Tu blocked on the ribosome by nonhydrolyzable GTP analogs (Haenni & Lucas-Lenard, 1968) or kirromycin (Wolf *et al.*, 1977) prevents the aa-CCA-end from participating in the peptidyl-transferase reaction. The overall similarity between the ternary complex and EF-G•GDP (see Section 3) termed 'macromolecular mimicry' (Nissen *et al.*, 1995) suggests common binding sites for these two complexes, in line with competition phenomena indicating that the ribosomal binding sites for EF-Tu and EF-G are at least partially overlapping (for references : Lucas-Lenard & Beres, 1974). This is also supported by the protection of the α -sarcin loop of ribosomal 23 S RNA by either EF-Tu or EF-G (Noller, 1991) and the common functional role of proteins L7/L12 in the activity of both factors (Sander *et al.*, 1973; Sander *et al.*, 1975). Direct mapping of the EF-G binding site on the ribosome has recently led to a model for EF-G binding in a position which indeed overlaps the observed position of the kirromycin-blocked ternary complex (Wilson & Noller, 1998). This and the different relative orientations of tRNAs on the ribosome has led to the speculation that EF-G and EF-Tu, though engaged in different stages of elongation, may have a similar mechanism inducing rotational movements of ribosome-bound tRNAs (Wilson & Noller, 1998). This function of EF-Tu and EF-G could be related to their 'macromolecular mimicry'. However, to uncover the relevance of this puzzling similarity further studies are required to visualize the binding site of EF-G on the ribosome and to reexamine in more detail the extent of the competition between EF-Tu and EF-G. In fact, EF-Tu and EF-G from *Pseudomonas fluorescens* may be able to bind simultaneously to ribosomes (Beres & Lucas-Lenard, 1973). Moreover, EF-1 α from mouse ascite cells was reported not to dissociate from the ribosome during the elongation cycle, thus allowing the simultaneous binding of EF-2 (Grasmuk *et al.*, 1977).

By analogy with other GTP binding proteins and the recent 3D model of the complex Ha-ras p21•p120-GAP334 (Scheffzek *et al.*, 1997), the ribosome should interact with the effector region of EF-Tu, but this has yet to be proved.

8. The mechanism of GTP hydrolysis

In the past years the intrinsic GTP hydrolysis of GBPs has drawn much interest, despite its low activity (see also: Hilgenfeld, 1995). After the finding that GTP hydrolysis by EF-Tu very likely takes place by an S_N2 in-line attack of a water molecule on the γ -phosphate (Eccleston & Webb, 1982), attention was directed towards EF-Tu residues potentially able to activate a nucleophilic water molecule located at the γ -phosphate, in the context of a general base catalysis. Candidate H84 was examined by mutagenesis (Cool & Parmeggiani, 1991; Scarano *et al.*, 1995; Zeidler *et al.*, 1995) and the results shed doubts on the validity of this hypothesis. Moreover, the EF-Tu_{Tt}•GMPPNP model revealed the existence of a 'hydrophobic gate' formed by the conserved residues V20 and I60¹, hindering the approach of the side chain of H84 to the nucleophilic water molecule (Berchtold *et al.*, 1993). Also in p21 water activation by the analogous residue Q61 was contested by studies concluding that the most basic moiety in the phosphate cavity and most probable proton acceptor in the reaction is actually the γ -phosphate itself (Langen *et al.*, 1992; Schweins *et al.*, 1994; Schweins *et al.*, 1995). A similar mechanism could also be implicated in the intrinsic GTPase of EF-Tu (Hilgenfeld, 1995). An alternative view postulates that the character of the transition state in phosphotransfer reactions is in most cases dissociative rather than associative, as it is for GTP hydrolysis in solution (Maegley *et al.*, 1996). Accordingly, protonation of the γ -phosphate would hinder the reaction, whereas the hydrogen bond from the main chain amide of the P-loop residue G13 (D21 in EF-Tu) to the β - γ -bridging oxygen, which has a strong NMR shift (Redfield & Papastavros, 1990; Lowry *et al.*, 1991), would considerably contribute to catalysis (Maegley *et al.*, 1996). Against this mechanism one can object that the character of the transition state in a biological system is not always the same as in solution, whereas in its favor one should mention that mutations of EF-Tu residues such as V20 (Jacquet & Parmeggiani, 1988), T25, I60 (I. M. Krab, unpublished) and H84 (Cool & Parmeggiani, 1991), expected to perturb the conformation of the phosphate cavity, have shown a corresponding decrease of the GTPase activity. In this regard, γ -phosphate-assisted hydrolysis should be less sensitive to conformational changes. Another factor that needs to be considered is the typical stimulation of the EF-Tu GTPase by mono- and divalent cations (Fasano *et al.*, 1978), much less pronounced in p21 (Mistou *et al.*, 1992), which remains without structural explanation.

If the mechanism for intrinsic catalysis presents unclear aspects, the ribosome stimulation of the GTPase of EF-Tu remains even more obscure. Individual ligands like aa-tRNA, empty ribosomes and kirromycin can partially activate its catalytic activity, but the full effect requires the concerted action of the programmed ribosome with peptidyl-tRNA in the P-site and a cognate aa-tRNA complexed to EF-Tu•GTP. By analogy with other GTPases (Bourne *et al.*, 1991; Moore *et al.*, 1993; Mittal *et al.*, 1996), two models should be considered. Either the ribosome accelerates a rate-limiting conformation change of EF-Tu toward the catalytic state or

¹ *E. coli* numbering of the equivalent residues used

alternatively the ribosome directly contributes to the configuration of the catalytic step. Recent structures of transition state mimicking complexes of GTPases with $\text{GDP}\cdot\text{AlF}_{3/4}$ and effectors have substantially advanced our understanding of effector-stimulated GTPase. Only heterotrimeric G_α subunits contain all elements necessary to form the transition state complex without the participation of an effector (Coleman *et al.*, 1994; Sondek *et al.*, 1994). The effector RGS4 further stabilizes the conformation of $G_i\alpha_1$ in this state without essential alterations (Tesmer *et al.*, 1997). In the small GTPases the GAP contributes an essential residue, the so-called ‘arginine finger’, to the catalytic site, neutralizing negative charges on the γ -phosphate. Further influence comes from stabilization of the switch regions by GAP, allowing ras Q61 to participate in the catalysis, playing an essential role by hydrogen bonding to the nucleophilic water (Rittinger *et al.*, 1997b; Scheffzek *et al.*, 1997). These models favor transition state stabilization as catalytic mechanism over water activation. Still, from the conformational difference seen between ‘ground-state’ complex $\text{Cdc42Hs}\cdot\text{GMPPNP}\cdot\text{RhoGAP}$ (Rittinger *et al.*, 1997a) and ‘transition state’ complex $\text{RhoA}\cdot\text{GDP}\cdot\text{AlF}_4\cdot\text{RhoGAP}$ the GTPase reaction may be preceded by a rate-limiting conformational change (Rittinger *et al.*, 1997b), as has been suggested for the GAP stimulation of p21 GTPase, a model that has elicited conflictual views (Neal *et al.*, 1990; Rensland *et al.*, 1991; Moore *et al.*, 1993). Also EF-Tu seems to undergo a conformational change on the ribosome prior to GTP hydrolysis (Rodnina *et al.*, 1995).

As in p21, in the EF-Tu ternary complex the β - γ -bridging oxygen of GTP is accessible from the outside, making ribosome-assisted catalysis via an amino acid or RNA base a possibility. So far, however, no isolated ribosomal protein has been shown to stimulate the GTPase activity of EF-Tu (Sander *et al.*, 1980) and R73 from protein L7/L12, a candidate for an ‘arginine finger’, is conserved in bacterial but not in the homologous eukaryotic proteins (Otaka *et al.*, 1990). One should also stress that the exact configuration of the transition state mimic of $\text{p21}\cdot\text{GAP}$ and $G_i\alpha_1$ models is not possible in EF-Tu, since in both cases it depends on the bonding properties of the critical Q61 and Q204, respectively, that in EF-Tu is a histidine (H84) (Hilgenfeld, 1995). Even if through the action of the ribosome the ‘hydrophobic gate’ were removed, allowing H84 to enter the catalytic site, it is probable that the mechanism for GTP hydrolysis in EF-Tu displays substantial differences from that of p21 or G_α , since interactions with multiple ligands are necessary for an efficient stimulation of the EF-Tu GTPase in the ‘burst’ GTP hydrolysis (Parmeggiani & Sander, 1981) and Section 4). The interactions inducing the optimum catalytic conformation of EF-Tu, implying the aa-tRNA CCA-end and a ribosomal region including the 50S α -sarcin loop, remain for the most part to be defined.

The mechanism by which empty 70S ribosomes (Parmeggiani & Sander, 1981; Jacquet & Parmeggiani, 1988), aa-tRNA (Parlato *et al.*, 1983) or kirromycin (Parmeggiani & Swart, 1985) alone can enhance the EF-Tu GTPase is not clear. It probably implies conformational changes around the nucleotide binding pocket, that influence the pKa of an acceptor group,

make the β - γ -phosphates a better leaving group or reorient residues affecting the transition state.

9. The elongation cycle revisited

In the last few years, the traditional model of elongation (see Section 1) has undergone some reelaboration, taking into account the flexibility of the ribosome during the elongation cycle. The hybrid states model (for references: Green & Noller, 1997), based on protection experiments, implies a dissociation of the movements of tRNA between the sites on the 30S (A and P) from the sites on the 50S subunit (A, P and E). In the initial binding of the ternary complex to the ribosome the codon-anticodon interaction takes place on the 30S A-site, while the CCA-end of tRNA is protected by EF-Tu, mainly bound to the 50S T-site (A/T state). After GTP hydrolysis and EF-Tu release the aa-CCA-end of tRNA enters the 50S A-site (A/A state, equivalent to the traditional A-site). The alternative allosteric three-site model is characterized by a stable codon-dependent binding of deacylated tRNA to the E-site and a reciprocal functional linkage between A- and E-site (Nierhaus, 1990). The characteristics of the two models are not incompatible (Weijland & Parmeggiani, 1994; Nierhaus *et al.*, 1997). Both encompass the traditional notion of a pretranslocational state interacting preferentially with EF-G and a posttranslocational state interacting with EF-Tu. Synergy between EF-Tu and EF-G in GTPase stimulation by empty ribosomes suggests that alternation between these basic conformations is an inherent property of ribosomes (Mesters *et al.*, 1994a).

Conflicting views still surround the energy consumed by EF-Tu on the ribosome, even after three decades. Most of the difficulties probably stem from the functional heterogeneity of purified ribosomes and the inborn properties of EF-Tu and EF-G GTPases to be triggered by ribosomes independently of elongation (Chinali & Parmeggiani, 1980). A total of two GTP molecules consumed vs amino acid incorporation per elongation cycle has found consensus mainly because EF-Tu and EF-G each can only bind one GTP (GDP) molecule. Obtained stoichiometries higher than the expected 2:1, such as 3 to 1, were considered to be due to uncoupled GTPase (Chinali & Parmeggiani, 1980). Also the 1:1 stoichiometry for GTP hydrolyzed:EF-Tu-mediated Phe-tRNA binding to the programmed ribosome was preferred to the unexpected 2:1 ratio that was observed now and then (Thompson *et al.*, 1981). In 1990, Ehrenberg *et al.* (Ehrenberg *et al.*, 1990) reported that a stable complex between EF-Tu•GTP and Phe-tRNA comprises not one but two EF-Tu molecules, forming a so-called pentameric complex (later renamed more correctly quinary (Bosch *et al.*, 1996) or extended ternary complex (Bilgin & Ehrenberg, 1995; Dinçbas *et al.*, 1995)). Two GTP molecules were hydrolyzed for each amino acid incorporated into the Poly(Phe) chain in a multi-round system lacking EF-Ts. These results were supported by a different approach (Weijland & Parmeggiani, 1993) in a complete system driven by EF-Tu[D138N]. This engineered EF-Tu uses xantine instead of guanine nucleotides (Hwang & Miller, 1987), otherwise showing the same

functional properties as wt EF-Tu (Weijland *et al.*, 1994). The XTP system, that eliminates any interference from EF-G-dependent GTPase or ribosome-bound nucleotidases, was also used to calculate a 3:1 stoichiometry for the total (EF-Tu + EF-G) energy consumption per elongation cycle (Weijland *et al.*, 1994). The existence of a quinary complex was contested by Bensch *et al.* (Bensch *et al.*, 1991), but supported by Rodnina and Wintermeyer (Rodnina & Wintermeyer, 1995) who however reported a 1:1 stoichiometry between GTP hydrolyzed and amino acid incorporation by analyzing step by step the synthesis of tripeptides dependent on hetero-mRNA-programmed ribosomes. Only when the adjacent Phe codons UUUUUC were translated in the presence of EF-G both GTP of their quinary complex were hydrolyzed, yielding a 2:1 stoichiometry restricted to the first Phe incorporated. They concluded that a 2:1 stoichiometry represents an exception related to the translation of a homopolymeric stretch of mRNA and the presence of EF-G, possibly connected with frameshift correction. Noteworthy, Ehrenberg *et al.* (Ehrenberg *et al.*, 1995) found a 2:1 stoichiometry using heteropolymeric mRNAs including one for which Rodnina and Wintermeyer report a 1:1 stoichiometry (Rodnina & Wintermeyer, 1995).

The importance of these aspects for the basic mechanism of elongation invites a detailed analysis. Against the existence of quinary complex is the lack of a convincing visualization of this complex by separation methods and of functional justifications. Moreover, no quinary complex has been revealed by crystallographic studies. As an even stronger argument, small angle neutron and X-ray scattering analysis of solutions containing EF-Tu, GTP and aa-tRNA (Bilgin *et al.*, 1998) have only revealed the existence of a ternary complex (as did others before: Leberman, 1995), close in structure to that of the crystallographic model. Thus, the existence of a stable quinary complex has become highly improbable, though transient interactions between ternary complex and EF-Tu GTP cannot be excluded.

Still open remains the stoichiometry between GTP hydrolysis and amino acid incorporation. A main reason for this is likely the *in vitro* system, in particular the difficulty of isolating fully active ribosomes after drastic high-salt washes as required to eliminate ribosome-bound nucleotidases. After purification, the fraction of functionally homogeneous, active ribosomes has been estimated to vary between <10 to 50 % of the total population (Wagner *et al.*, 1982; Bartetzko & Nierhaus, 1988); A. Parmeggiani, unpublished). Isolation of ribosomes 100 % active in elongation after high-salt washes was claimed in only one case (Rodnina & Wintermeyer, 1995). That two EF-Tu molecules can functionally cooperate for a coordinated action on the ribosome has been shown by genetic and biochemical studies on a mixed population of EF-Tu mutants (see Section 3.3). Several functional reasons can be invoked for a 2:1 stoichiometry (Weijland & Parmeggiani, 1994). It can be connected with an increased accuracy in translation: two sequential EF-Tu•GDP releasing steps could allow the ribosome to build up a higher intrinsic accuracy, according to the theoretical model of Hopfield (Hopfield, 1974), but this remains to be experimentally proved. Another possibility can be derived from

the three-site model of the ribosome, according to which the release of the deacylated tRNA from the E-site would facilitate the accessibility to the A-site (Nierhaus, 1990). One EF-Tu•GTP could induce the ejection of deacylated tRNA from the E-site, the second being consumed for the correct positioning into the A-site. In fact, in fungi a third essential elongation factor (EF-3) was reported to facilitate the release of deacylated tRNA from the E-site (for references: Triana-Alonso *et al.*, 1995). So far, however, no experimental evidence has confirmed this possibility in the *E. coli* system. Moreover, the allosteric correlation between E- and A-sites is controversial (Kirillov & Semenov, 1986; Semenov *et al.*, 1996; Nierhaus *et al.*, 1997). The possibility that a 2:1 stoichiometry is connected with frame-shift correction with UUU codons (Rodnina & Wintermeyer, 1995) is difficult to envisage, given the precise 2:1 ratio on long poly(U) stretches. An indirect evidence for the interaction of the ribosome with two EF-Tu•GTP molecules is the observation that EF-Tu requires two L7/L12 pairs for optimal ribosome-dependent GTPase and poly(Phe) synthesis activity, whereas for EF-G one pair is sufficient (Möller *et al.*, 1983). Another possible explanation, for the 2:1 stoichiometry is the activity of EF-Tu as chaperon (see Section 10).

In this context, Bosch *et al.* (Bosch *et al.*, 1996) have proposed a revised model for the elongation cycle, based on the observed cooperativity between two EF-Tu mutants (see Section 3.3). Noncognate ternary complexes would induce via ribosome-elicited GTP hydrolysis a highly selective A-site restricted to cognate and near-cognate ternary complexes. A second ternary complex would insert the aa-tRNA in the A-site depending on codon-anticodon interaction, expending another GTP. Problems with this scheme are the lower GTPase activity induced by the ribosome in the absence of codon-anticodon interaction (Swart *et al.*, 1987; Jacquet & Parmeggiani, 1989; Rodnina *et al.*, 1996), at least in the *in vitro* conditions used so far, and the uncertainty in extending observations with mutant EF-Tu's to the wild-type system. However, this model represents an interesting effort to integrate *in vivo* and *in vitro* results.

In conclusion, possible specific functions of two EF-Tu•GTP in elongation as well as the basis of their intermolecular interactions remain to be clarified. None the less the hydrolysis of two GTP molecules appears to be compatible with most of our present functional/structural knowledge of ribosomes and EF-Tu, as a result of a coordinated interaction between ribosome, ternary complex and a second EF-Tu•GTP, without implying the formation of a stable quinary complex.

10. A new development: EF-Tu as chaperon

Recently, *in vitro* findings have shown that EF-Tu_{Tt} can act as a chaperon on the renaturation of urea-denatured rhodanese (Kudlicki *et al.*, 1997), a model protein extensively used to study protein folding. The unassisted refolding of rhodanese is a slow process that is markedly accelerated by specific chaperons or 50S ribosomal subunits and 23S RNA (for references: Kudlicki *et al.*, 1997). Nucleotide-free EF-Tu_{Tt} can promote a partial renaturation of 1:1

stoichiometric amounts of rhodanese, but its action becomes much more efficient when in complex with GTP and in the presence of EF-Ts, in conditions inducing multiple rounds of GTP hydrolysis. This effect was observed to depend on the EF-Tu_{Tt} transitions leading to the 'off'- and 'on'-states, taking place during GTP hydrolysis and the GDP/GTP exchange, respectively. Accordingly, the chaperon-like activity is reduced by freezing the two basic states with kirromycin and pulvomycin. In favor of a broad action as chaperon is the evidence that EF-Tu_{Ec} can promote a productive folding of other denatured proteins such as citrate synthase and α -glucosidase, associates with unfolded proteins and protects native proteins against heat-denaturation (Caldas *et al.*, 1998). Moreover, these authors observed that EF-Tu displays disulfide isomerase activity, facilitating the correct redox status of nascent proteins (G. Richarme, personal communication). Even though EF-Tu was not mentioned as one of the proteins associated to the nascent β -galactosidase using an *E. coli* transcription/translation system (Hesterkamp *et al.*, 1996), it is tempting to take into consideration that EF-Tu may somehow contribute to induce the correct folding of the nascent polypeptide chain on the ribosome, with a mechanism involving a GTP hydrolysis independent from that associated to the binding of the ternary complex to the A-site. This activity could represent a further alternative for explaining the consumption of two molecules of EF-Tu•GTP in poly(Phe) synthesis. To date this possibility is speculative and additional data are required for the physiological significance of this novel finding, but the EF-Tu-assisted refolding could be promising for innovative developments.

11. Conclusions

Thanks to the impressive progress of our knowledge of the 3D structure of EF-Tu and its complexes it is now possible to analyze the structural background of most partial activities of this factor. This allows structural dynamic studies directed to analyze the transitional changes taking place during the various interactions. In fact, the existing models only represent 'snapshots' of the complex dynamics of the functional cycle of EF-Tu. A major challenge remains the interaction between EF-Tu and the ribosome, of which our knowledge still lies in the initial phase due to its complexity. The recent visualization of EF-Tu on the ribosome may soon allow a better targeted approach for the study of the ribosomal elements involved in this binding. The possible physiological role of the molecular mimicry between EF-G•GDP and the ternary complex of EF-Tu merits a closer examination. Conflicting views on the function of EF-Tu in elongation, particularly its energy consumption, still remain to be solved. Other long known aspects such as the reason for the existence of two *tuf* genes and their regulation require further examination. The newly discovered ability of EF-Tu to assist protein (re)folding may have an impact on our understanding of its cellular functions. Even 30 years after its discovery EF-Tu still remains a source of exciting developments and represents a model for GTPases and allosteric regulation.

12. Summary

Elongation factor Tu (EF-Tu), the carrier of aa-tRNA in protein biosynthesis, has been a model protein nearly since its discovery three decades ago. It was the first GTPase of which specific conformational changes were unveiled. To date the 3D structures of both GDP and GTP-bound states and its complexes with aa-tRNA and EF-Ts have been solved, its location on the ribosome has been visualized at low resolution and the interactions with aa-tRNA, EF-Ts, ribosomes and antibiotics are being explored at the molecular level with a variety of techniques. Mutagenesis has been essential for determining structure-function relationships of the nucleotide binding pocket, the three domains and the interaction with ligands. Despite the considerable progress, basic questions still await an unequivocal answer. Among them, the mechanism of GTP hydrolysis, especially in its fast version induced by codon-anticodon interaction. Here, EF-Tu may display features different from those of other GTPases. Also the energy consumption in the EF-Tu cycle on the ribosome represents an unsolved problem. Interesting perspectives arise by a novel finding, its ability to enhance protein folding. This review reports on these diverse aspects of EF-Tu, underlining the most recent developments and proposing possible explanations.

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Chapter 2b

Some more information
from recent literature

3D structures of EF-Tu

A new and higher resolution structure (2.05 Å) of antibiotic-free intact EF-Tu•GDP from *E. coli* (PDB code: 1efc, Song *et al.*, 1999) shows in more detail the interactions of water molecules around the Mg²⁺-GDP complex. Also some side-chain interactions, like that of Arg58, are shown to be different from that in the *T. aquaticus* structure, meaning that their interactions are probably not structurally very important. A very recent contribution is that of bovine mitochondrial EF-Tu at 1.94 Å resolution (Andersen *et al.*, 2000). It displays a different orientation of domain 1 relative to the other two domains as compared to the EF-Tu structure. A C-terminal extension shows structural similarity to zinc-finger proteins, suggesting that it may be involved in the recognition of RNA. The lower affinity for nucleotides of mtEF-Tu compared to the bacterial one is ascribed to a possibly increased flexibility of elements surrounding the binding site. A second ternary complex structure, that of EF-Tu_{Tt}•GDPNP•Cys-tRNA^{Cys}_{Ec} confirms the general principles of EF-Tu-tRNA interaction that were derived from the Phe-tRNA^{Phe} ternary complex while specific differences in the way the 3'-aminoacyl ends are bound suggest how all 20 different aminoacylated 3'-ends may be accommodated (PDB code: 1b23, Nissen *et al.*, 1999). Most interestingly, the first complex of EF-Tu bound to a specific antibiotic, GE2270A, has been resolved to 2.35 Å (Heffron & Jurnak, 2000). It shows the antibiotic bound solely to domain 2 of the GDP-bound form of EF-Tu. Extrapolation to the GTP-bound structure places the ring structure of GE2270A in the domain 1-2 interface, where it would cause a clash with β-strands from the core and α-helix 2, thus hindering the formation of the normal compact GTP-bound structure. It moreover would occupy the space accommodating the aminoacyl end of aa-tRNA, in agreement with this antibiotic's inhibitory action on ternary complex formation. A salt bridge between the non-conserved residues Arg223 and Glu259 closes over the antibiotic and may explain the very strong interaction with EF-Tu making removal, once bound, practically impossible.

Ribosome structure

Exciting progress has recently been made concerning structural studies of the ribosome. Crystal structures of the *Thermus thermophilus* 30S subunit at 5.5 Å (Clemons Jr *et al.*, 1999), the *Haloarcula marismortui* 50S subunit at 5 Å (Ban *et al.*, 1999) and the *Thermus thermophilus* 70S ribosome with tRNAs bound to different combinations of ribosomal sites at up to 7.8 Å resolution (Cate *et al.*, 1999) were published at the end of 1999. A cryoelectron microscopy (cryo-EM) study of the large subunit achieved 7.5 Å resolution (Matadeen *et al.*, 1999) and shows some of the dynamic structural changes in high detail. The entire 50S ribosome was resolved to 10.5 Å already with this technique (Gabashvili *et al.*, 2000). At the April 2000 tRNA meeting in Cambridge a crystallographic density map of the 50S subunit at 2.7 Å was announced (Nissen *et al.*, 2000).

These successes allow direct visualization of many rRNA helices and proteins. Those

ribosomal proteins of which a high-resolution structure is known could be placed in a detailed context on the ribosome, and others can be seen for the first time at medium resolution (Ban *et al.*, 1999; Clemons Jr *et al.*, 1999). A 2.7 Å resolution as announced for the 50S subunit would even allow to model the protein structures directly.¹ As an answer to a long-standing question, from the modelling of the substrate analogue CCdA-puromycin into their model Nissen *et al.* deduce that the ribosome is a ribozyme in which rRNA catalyzes the peptidyl transfer (Nissen *et al.*, 2000). Indeed, the intersubunit areas consist primarily of RNA, while proteins mostly form an outer shell on the rRNA skeleton. On the 30S subunit a striking feature is the penultimate helix of the 16S rRNA, which harbours the “decoding site”. This runs the length of the body of the 30S on the intersubunit interface, forming some of the most important subunit contacts. It may play a role in transmitting conformational changes to the rest of the ribosome, like those associated with alternative pairings of bases 910-912 adjacent to the 16S rRNA central pseudoknot (Gabashvili *et al.*, 1999). Interestingly, ribosomes isolated from cells in which growth was arrested by kirromycin treatment (thus containing mostly “jammed ribosomes” on the polysome) have a more compact conformation as shown by cryo-EM (Zhang *et al.*, 1998). These authors find that cells starved for tryptophan (thus for aa-tRNA) on the other hand, have the more open conformation that is now being elucidated at high resolution. Noteworthy, their results suggest that most ribosomes in exponentially growing cells are in the compact conformation, meaning that caution should be exercised in interpreting the ribosome’s mechanism of function from the current set of structures.

Ribosomal sites interacting with elongation factors

Structures of fragments of the 50S subunit that interact with the elongation factors have been resolved at high resolution, like the isolated ribotoxin loop (also called α -sarcin loop) (Correll *et al.*, 1998; Correll *et al.*, 1999) and the complex of protein L11 and the underlying region of 23S rRNA (Wimberly *et al.*, 1999), and could be fitted into the new high resolution ribosome structures. Both sites are implicated in conformational switches. Noteworthy, the concerning parts of 23S rRNA bind to EF-G free in solution (Munishkin & Wool, 1997). Although for EF-Tu this has not been shown, several synthetic RNAs selected for binding to EF-Tu show a consensus motif also found in the ribotoxin region (Hornung *et al.*, 1998). They, however, do not bind to EF-G. Thus it is possible that these RNAs mimic a conformation of the ribotoxin region that it can achieve only in the context of the pre-translocation state of the ribosome that has affinity for EF-Tu. Also the ribosomal stalk and especially L7/L12 are the focus of continued attention. Even if without L7/L12 some factor-dependent amino acid incorporation was described (e.g. Koteliansky *et al.*, 1977), fast protein synthesis requires an intact stalk (Pettersson & Kurland, 1980). A single-headed dimer of L7/L12 was recently found to be sufficient (Oleinikov *et al.*, 1998). Whereas the EF-Tu GTPase is not stimulated by free

¹ In the mean time this structure has been refined to 2.4 Å and was published in Nature (Wimberly *et al.*, 2000), while a 3.0 Å structure of the 30S subunit has been published in Science (Ban *et al.*, 2000)

L7/L12 (Sander *et al.*, 1980), a recent report finds a stimulation of EF-G's GTPase at high concentrations ($K_M = 43 \mu\text{M}$), and by mutagenesis confirms that the single arginine residue in the head is not important for this stimulation (Savelsbergh *et al.*, 2000). Mutation of this residue also had no or little influence on translational activity in *Sulfolobus solfataricus* ribosomes, whereas mutating some conserved aromatic residues or deleting a highly charged region in the head, did (Kusser *et al.*, 1999). Thus this arginine is unlikely to play the role of an "arginine finger" in a GAP-like action of L7/L12 on elongation factors. An interaction was also reported between the eukarotic counterparts eEF2 and P proteins (Bargis-Surgey *et al.*, 1999), but unfortunately no functional effect of this interaction was shown. More interestingly, replacing L7/L12 by the eukaryotic P complex yields hybrid ribosomes that stimulate the GTPase of eEF2, but no longer that of EF-G (Uchiumi *et al.*, 1999).

Visualization of elongation factor interactions with the ribosome

Cryo-EM studies increasingly also reveal the interactions of the ribosome with elongation factors. After the kirromycin-stalled EF-Tu•GDP•aa-tRNA complex on the ribosome (Stark *et al.*, 1997), also fusidic acid-stalled EF-G on the empty ribosome ("post-translocational" complex) was visualized (Agrawal *et al.*, 1998), followed by higher-resolution structures of the same on programmed ribosomes and of EF-G•GMPPCP on both empty and programmed ribosomes (pre-translocational complex) (Agrawal *et al.*, 1999). Another approach used the antibiotic thiostrepton, which strongly slows down translocation and turn-over GTPase stimulation of EF-G by ribosomes, without decreasing the kinetics of first-round gamma phosphate cleavage (Rodnina *et al.*, 1999). Thus, a "post-hydrolysis pre-translocational" complex was resolved showing a totally different orientation of EF-G from that using a non-cleavable GTP analog, as well as an alternative post-translocational structure in which the relative orientation of the EF-G domains differs, especially concerning the interaction with the base of the stalk (Stark *et al.*, 2000). However, it should be remembered that these complexes are artificially stalled using antibiotics which hamper normal functioning. For example, thiostrepton binds to and interferes with just the region at the base of the stalk (see also: Ban *et al.*, 1999) that Stark *et al.* find not to interact with EF-G in their alternative post-translocational complex. Also the structure of EF-Tu•GDP•kirromycin•aa-tRNA complex on the ribosome needs to be interpreted with caution and comparison with a ternary complex with a non-hydrolyzable GTP analog would be informative, because a crosslinking study found that binding of a GMPPNP-containing ternary complex, but not a GDP/kirromycin ternary complex increases crosslinking from specific positions of L7/L12 to several ribosomal proteins (Dey *et al.*, 1998).

Fast kinetics studies of elongation factor interactions with the ribosome

Kinetic parameters for all the reaction steps of the elongation have now been compiled by

assembling fast kinetics measurements of partial reactions and global fitting (Pape *et al.*, 1998). The initial steps in the EF-Tu-dependent part of the cycle are all relatively fast, but the dissociation of EF-Tu•GDP from the ribosome is found to be the slowest, thus rate-limiting, while aa-tRNA accommodation is only slightly faster. The measurements were done at two different Mg^{2+} concentrations in the absence of polyamines. Some rates, especially that of the GTPase activation and GTP hydrolysis step, were found to depend on the Mg^{2+} concentration, being almost five times faster at 10 mM than at 5 mM. The low rates (3 s^{-1}) of the slowest step still does not account for the 10-20 aa/s elongation speed that can be achieved *in vivo*, but this probably reflects the different ionic and buffer conditions used *in vitro*, the fact that single steps using nucleotide analogs etc. were measured, and perhaps that the purification procedure alters the ribosomes in unknown ways, decreasing their efficiency. Also the mechanism of proofreading has been studied using this fast kinetics approach on the binding of a near-cognate aa-tRNA (Pape *et al.*, 1999). It appears to be a compound effect of (compared to binding of a cognate aa-tRNA) an 85-fold higher reverse rate of the codon recognition step, a ten-fold lower GTPase activation rate (compared to a 10^4 reduced rate for a non-cognate aa-tRNA (Rodnina *et al.*, 1996)) and a 70-fold decreased rate of aa-tRNA accommodation after EF-Tu release, resulting in at least 20-fold increase in aa-tRNA rejection. Thus at each of several steps a near-cognate is “discouraged” from proceeding compared to a fully cognate aa-tRNA. The authors propose an “induced fit” mechanism of the recognition, in which a cognate aa-tRNA more efficiently induces a conformation of the ribosome that triggers the EF-Tu GTPase and accommodates the aa-tRNA in the A-site. The question of the coupling of the codon recognition signal to the GTPase activation was also examined by an approach dividing the aa-tRNA into two fragments, an A-site binding anticodon/D-loop (ACD) fragment and an EF-Tu binding acceptor stem/T hairpin fragment (Piepenburg *et al.*, 2000). This “split aa-tRNA” is not able to trigger EF-Tu GTPase hydrolysis on A-site binding like an intact aa-tRNA•EF-Tu•GTP complex would. This shows that a cognate codon-anticodon interaction of the anticodon stem-loop by itself does not generate in the ribosome the signal for activating the GTPase trigger, but unfortunately, it does not allow to decide whether the trigger signal passes through the aa-tRNA, which therefore must be intact, or whether the entire aa-tRNA on codon-anticodon interaction induces a conformation in the ribosome that causes the trigger on EF-Tu.

Subcellular localization

EF-Tu has over the years been found to be associated with either the membrane fraction or the periplasm of the cells. Recently it was found that upon osmotic downshock EF-Tu is one of a few proteins that are transferred to the periplasm, a process in which mechanosensitive channel mcsL was implicated (Berrier *et al.*, 2000). This finding means that caution should be exercised when isolating cells to avoid inadvertent effects. For example, the finding of EF-Tu in the periplasm of *Neisseria gonorrhoeae* (Porcella *et al.*, 1996) may thus be an artefact. The

few cases of membrane-association reported for *E. coli* EF-Tu occur in exceptional (stress) circumstances, induced by a respiratory uncoupler (Sedgwick & Bragg, 1986), growth in absence of sucrose of a sucrose-dependent mutant (Dombou *et al.*, 1981), or nutrient-limitation inducing EF-Tu methylation as well (Young & Bernlohr, 1991). In *Mycobacterium leprae* EF-Tu was found in the membrane fraction in the absence of particular stress (Marques *et al.*, 1998).

Other roles of EF-Tu

The all but forgotten possible role of EF-Tu in regulation of transcription has received a new impulse with the recent finding that EF-Tu interacts directly with the C-terminal region of the beta subunit of RNA polymerase (Trigwell & Glass, 1998). The authors suggest a possible role of EF-Tu in stringent response. Further experimental examination of the functional implications should clarify this point. The possible role of EF-Tu as a chaperon protein has been enlarged by a study showing that it possesses an exceptionally reactive cysteine residue, lending it a protein disulfide isomerase activity (Richarme, 1998). Moreover, both EF-G and IF2 were shown to have properties similar to those found for EF-Tu concerning stimulation of protein refolding and binding to denatured proteins, thus showing that EF-Tu is not unique in having these properties usually ascribed to chaperon proteins (Caldas *et al.*, 2000). What the physiological meaning is of this remains to be shown.

EF-Tu genetics and antibiotics-related findings

As already mentioned in the previous chapter, either *tuf* gene in *E. coli* has been shown to be dispensable (Zuurmond *et al.*, 1999b). Moreover, a strain containing a single *tuf* gene modified to code for a C-terminally His₆-tagged EF-Tu could be created. This allows for easy preparation of EF-Tu-free cell extracts using nickel-NTA agarose for in vitro translation experiments with modified EF-Tus. Interestingly, an *tufA* mutant selected for reversal of streptomycin dependent phenotype (caused by S12 mutation) turned out to code for EF-Tu[A375T]. Elimination of *tufB* from this strain severely hampered growth, but a *tufB*₀ allele (coding for the translationally inactive EF-Tu[G222D]) could reverse this impairment. This is yet another experimental contribution showing a cooperative effect on translation by two *tuf* genes, even if one of the products does not by itself sustain protein synthesis. Kirromycin-resistant EF-Tu mutants were also shown to be resistant (Zuurmond *et al.*, 1999a) to the new antibiotic enacyloxin IIa, whose effects on EF-Tu are characterized in *Chapter 7* of this thesis. These authors also contradicted the earlier finding (Cetin *et al.*, 1996) that enacyloxin IIa could inhibit C-terminal incorporation of Phe after non-enzymatic binding to the A-site of the ribosome and found in a mixed system dominance of enacyloxin IIa sensitivity over resistance, like for kirromycin, suggesting a “ribosome jam” type of inhibition mechanism (see also *Chapter 7*).

Chapter 3

Functional-structural analysis of threonine 25, a residue coordinating the nucleotide-bound magnesium in elongation factor Tu

Ivo M. Krab and Andrea Parmeggiani
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ABSTRACT

Elongation factor (EF) Tu Thr-25 is a key residue binding the essential magnesium complexed to nucleotide. We have characterized mutations at this position to the related Ser and to Ala, which abolishes the bond to Mg^{2+} , and a double mutation, H22Y/T25S. Nucleotide interaction was moderately destabilized in EF-Tu(T25S) but strongly in EF-Tu(T25A) and EF-Tu(H22Y/T25S). Binding Phe-tRNA^{Phe} to poly(U)•ribosome needed a higher magnesium concentration for the latter two mutants but was comparable at 10 mM $MgCl_2$. Whereas EF-Tu(T25S) synthesized poly(Phe) as effectively as wt, the rate was reduced to 50% for EF-Tu(H22Y/T25S) and was, surprisingly, still 10% for EF-Tu(T25A). In contrast, protection of Phe-tRNA^{Phe} against spontaneous hydrolysis by the latter two mutants was very low. The intrinsic GTPase in EF-Tu(H22Y/T25S) and (T25A) was reduced and the different responses to ribosomes and kirromycin suggest that stimulation by these two agents follows different mechanisms. Of the mutants, only EF-Tu(T25A) forms a more stable complex with EF-Ts than wt. This implies that stabilization of the EF-Tu•EF-Ts complex is related to the inability to bind Mg^{2+} , rather than to a decreased nucleotide affinity. These results are discussed in the light of the three-dimensional structure. They emphasize the importance of the Thr-25- Mg^{2+} bond, although its absence is compatible with protein synthesis and thus with an active overall conformation of EF-Tu.

INTRODUCTION

Elongation factor (EF) Tu belongs to the superfamily of guanine nucleotide-binding proteins, factors involved in numerous cellular processes as carrier of information or biological components. Common characteristics in the family are the switching between active and inactive conformations depending on whether GTP or GDP is bound (Bourne *et al.*, 1990; Bourne *et al.*, 1991), an intrinsic GTPase activity that can be stimulated several orders of magnitude by specific factors (GTPase-activating proteins), and the property that the GDP/GTP exchange can be enhanced by specific exchange factors (guanine nucleotide exchange factors) (reviewed in: Boguski & McCormick, 1993; Sprang, 1997). EF-Tu acts as carrier of aminoacyl-tRNAs (aa-tRNAs) to the ribosome in the elongation phase of bacterial protein biosynthesis. It has a long history of functional and structural studies offering many insights into its functioning (Krab & Parmeggiani, 1998). Structures of the two basic states of EF-Tu (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993; Abel *et al.*, 1996; Polekhina *et al.*, 1996) and of the complexes with its guanine nucleotide exchange factor EF-Ts (Kawashima *et al.*, 1996; Wang *et al.*, 1997) and with its “load” aa-tRNA (Nissen *et al.*, 1995) are now available. These provide snapshots of the dynamics of the factor during its cycle, which involves large interdomain rearrangements. On the other hand, many functional questions, such as the mechanism of GTP hydrolysis, the EF-Tu cycle on the ribosome, and the structure-function relationships of many structural elements, still remain unanswered or are awaiting satisfactory answers.

Domain 1 of EF-Tu (G domain) has the guanine nucleotide-binding protein conserved fold and contains the “fingerprint” conserved sequence motifs taking care of the interaction with the nucleotide (Bourne *et al.*, 1991; Kjeldgaard *et al.*, 1996). Each of the first three consensus motifs contains a residue involved in the coordination network of the essential magnesium ion interacting with the β - and γ -phosphates of the nucleotide. In *Escherichia coli* EF-Tu, these residues are Thr-25 and Thr-61, which bind to the magnesium ion directly via the OH-oxygen, and Asp-80 and Asp-50, which hydrogen-bond each to one of the water molecules, completing the coordination sphere of magnesium. In other guanine nucleotide-binding proteins, threonine from the first consensus element is often substituted by a serine, which can coordinate similarly to magnesium. The important role of this metal ion in the nucleotide binding is emphasized by the observation that its chelation decreases the affinity for GDP and GTP by at least 3 orders of magnitude (Miller & Weissbach, 1977). Significant amounts of free magnesium are not necessary for GTPase activity, and the tightly bound magnesium ion must be extracted with a large excess of EDTA before activity is lost (Ivell *et al.*, 1981; Mistou *et al.*, 1992).

EF-Tu Thr-25 is central in this coordination of magnesium, whereas Thr-61 located on the opposite side of the magnesium ion with respect to Thr-25 is only coordinated in the GTP form (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993; Abel *et al.*, 1996; Polekhina *et al.*, 1996).

Moreover, the homologue of Thr-61 in Ha-Ras p21, Thr-35, was found to only weakly interact with the ion in the GTP-bound form (Halkides *et al.*, 1994; Farrar *et al.*, 1997). On the other hand, mutation of p21 Ser-17 (the homologue of EF-Tu Thr-25) can be associated with dominant negative phenotypes, emphasizing its functional importance (Feig & Cooper, 1988; Farnsworth & Feig, 1991; Stacey *et al.*, 1991; Créchet *et al.*, 1996).

Because in EF-Tu the structure-function relationships of the residues involved in magnesium coordination are still mostly unexplored, we have analyzed in well defined *in vitro* conditions the effects associated with the substitution of Thr-25 with two closely related amino acids, serine and alanine. Serine is the amino acid functionally most similar to threonine; this substitution thus minimizes the changes induced in the magnesium ion network. The replacement with alanine emphasizes the consequences of suppressing the link to the magnesium ion. A double mutant EF-Tu(H22Y/T25S) isolated during the course of this study was also characterized and found to display intermediary properties, thus allowing to distinguish more precisely between specific effects of the two single substitutions.

MATERIALS AND METHODS

Biological materials

Ribosomes, EF-G, Phe-tRNA synthetase, and partially purified tRNA^{Phe} (5 to 11%) were prepared from *E. coli* as reported (Chinali *et al.*, 1977; Parmeggiani & Sander, 1981). Highly purified tRNA^{Phe} was also obtained from Sigma.

Construction, overproduction and purification of EF-Tu mutants

To obtain rapid purification, EF-Tu wt and mutants were overproduced as fusion with glutathione S-transferase (Smith & Johnson, 1988). Plasmid pGEX-2TtufA is a derivative of pGEX-2T (Smith & Johnson, 1988) containing the *E. coli tufA* gene coding sequence contiguous to the thrombin recognition sequence LVPRGS, where serine represents the first amino acid of EF-Tu (Anborgh, 1993). The very efficient cleavage by thrombin after arginine results in EF-Tu containing an additional N-terminal glycine, as compared with the native cellular EF-Tu, the N terminus of which is acetylated (Arai *et al.*, 1980). All mutant proteins and also the control wt used in this study were produced in this way. Mutant T25A was generated by means of the polymerase chain reaction. Mutant T25S was obtained by the Unique Site Elimination method (Deng & Nickoloff, 1992), using a kit from Amersham Pharmacia Biotech. Interestingly, when trying to construct the mutant T25S using either the polymerase chain reaction or Unique Site Elimination methods with an 18 base mutagenic primer, we systematically obtained a double mutant H22Y/T25S resulting from the substitution of a flanking base. Use of a longer primer of 25 bases including codon 22 allowed us to obtain

T25S alone.

The EF-Tu mutants were overproduced in *E.coli* DH5 α grown in LB medium at 30 °C to 0.5-0.8 A_{600} , after which induction with 0.08 mM isopropyl- β -D-thiogalacto-pyranoside took place at 22 °C with incubations up to 40 hrs. Cells were disrupted by sonication in 50 mM Tris-HCl, pH 7.5, 110 mM KCl, 10 mM MgCl₂, 7 mM ME and 10% glycerol (Buffer A). After centrifugation at 30,000 $\times$ g for 20 min, the supernatant was applied to a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech; 2.5 ml of resin /g cells), which was then extensively washed at 4 °C with Buffer A containing 30-150 μ M GDP, to remove bound EF-Ts as far as possible. Glutathione S-transferase-EF-Tu was cleaved *in situ* and eluted at 4 °C with Buffer A supplemented with 30 μ M GDP, 2.5 mM CaCl₂, and 1 unit/ml thrombin at a low flow rate (approximately 0.3 ml/min) until no more protein was released. Thrombin in the eluent was inactivated by adding 0.1 mM phenylmethylsulfonyl fluoride. The obtained EF-Tu was further purified to remove residual EF-Ts, using either Q Sepharose chromatography or Superdex 75 gel filtration. For the first method, the eluate from the glutathione affinity column was brought to a final GDP concentration of 250 μ M and applied to a Q Sepharose FF column (6 ml). This was washed with Buffer A containing 250 μ M GDP to induce the dissociation of EF-Tu•EF-Ts, and 120 mM KCl, at which concentration EF-Ts cannot bind to the resin. EF-Tu was eluted with a linear KCl gradient to 400 mM. For the second method, the affinity column eluate was concentrated by ultrafiltration (Amicon CentriFlo 25) to <2 ml before loading on an Amersham Pharmacia Biotech Superdex 75 column, which was eluted with Buffer A containing 40 μ M GDP. Pooled peak fractions from Q Sepharose or Superdex columns were concentrated by ultrafiltration, dialyzed against 50 mM Tris-HCl, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 1 mM dithiothreitol, 55% glycerol, 20 μ M GDP and stored at -30 °C. Protein concentrations were determined by the Bio-Rad protein assay, using bovine serum albumin as standard.

Functional assays

Poly(Phe) synthesis was determined kinetically from the formation of hot trichloroacetic acid-insoluble material (Swart *et al.*, 1987) and the GTPase activity was determined from liberation of [³²P]P_i using the molybdate method (Parmeggiani *et al.*, 1971). Nucleotide dissociation rates and affinities (Ivell *et al.*, 1981; Jensen *et al.*, 1989; Anborgh *et al.*, 1991; Cool & Parmeggiani, 1991), and protection of aa-tRNA by EF-Tu against spontaneous hydrolysis (Anborgh & Parmeggiani, 1993) were measured as described. Details are reported in the legends to the figures.

RESULTS

Production and purification of EF-Tu mutants

Glutathione S-transferase-fused EF-Tu was expressed in *E.coli* DH5 α cells grown in LB medium at 30 °C to 0.5-0.8 A_{600} . At this cell concentration and temperature, induction by 0.08 mM isopropyl- β -D-thiogalactopyranoside for 6-8 h resulted in high level overproduction. However, whereas overproduced glutathione S-transferase-EF-Tu wt and T25S displayed good solubility (70-80%), yielding up to 2 mg thrombin-cleaved EF-Tu/g cells (wet weight), mutants T25A and H22Y/T25S were about 10% soluble. Longer induction times (up to 40 hrs) at lower temperature during the induction phase (22 °C) somewhat increased the solubility. On the glutathione affinity column some EF-Ts is always retained as a complex with EF-Tu, making a second purification step necessary, either by Q Sepharose chromatography or Superdex 75 gel filtration (see under “Materials and Methods”). This was particularly the case for the Ala mutant,

which complexes very tightly with EF-Ts (75% EF-Tu•EF-Ts complex was still present after the first affinity step).

The presence of an additional glycine at the N-terminal extremity in EF-Tu wt did not modify its function in any of the activities tested in this work, including poly(Phe) synthesis.

TABLE I
Nucleotide interaction parameters

Shown are apparent rate constants for dissociation (k_{off}) and association (k_{on}) of GDP and GTP and the equilibrium dissociation constants (K_d) measured at 0 °C.

	GDP			GTP		
	$10^4 k_{\text{off}}$	$10^{-4} k_{\text{on}}^a$	K_d	$10^4 k_{\text{off}}$	$10^{-4} k_{\text{on}}^a$	K_d
	s^{-1}	$s^{-1} M^{-1}$	μM	s^{-1}	$s^{-1} M^{-1}$	μM
wt	3.9	56	0.00070	81	0.46	1.78
T25A	270	1.3	2.1	ND ^b	ND ^b	ND ^b
H22Y/T25S	108	1.1	1.0	350	0.34	10.4
T25S	10	48	0.0021	253	3.4	0.75

^a Calculated as $k_{\text{on}} = k_{\text{off}}/K_d$ assuming a two-state association/dissociation reaction without intermediate steps.

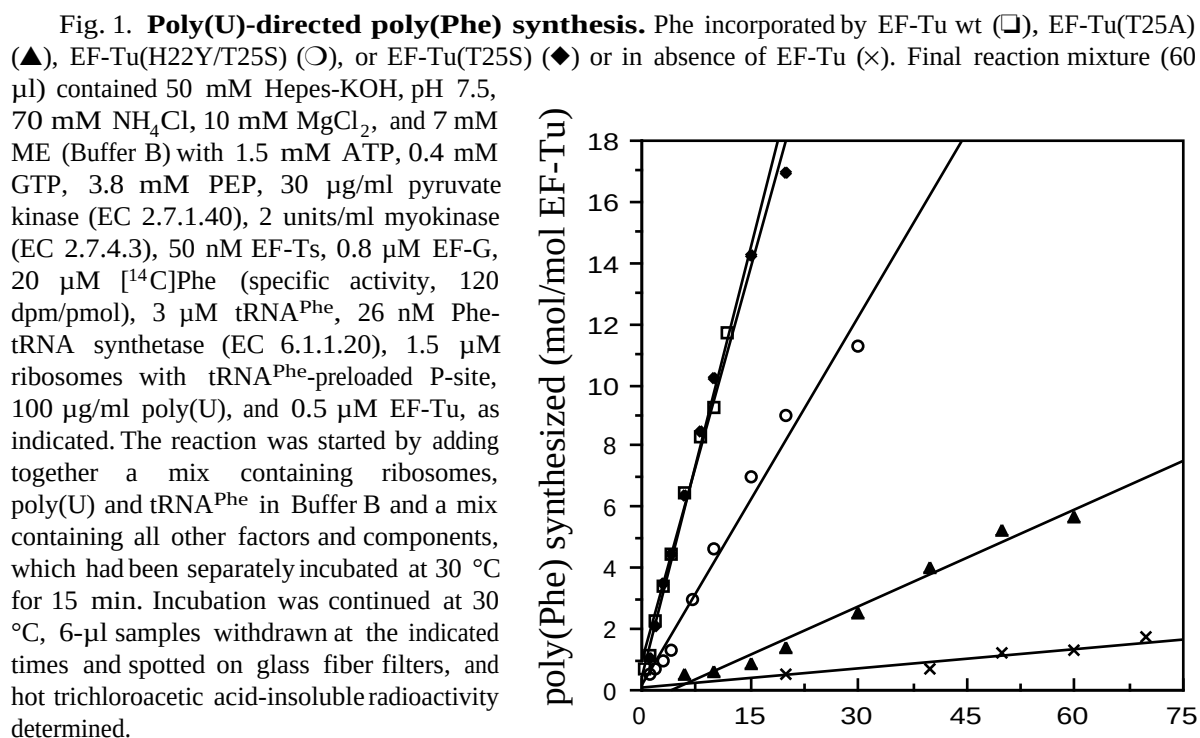
^b Could not be determined (ND) by the nitrocellulose binding method.

Interaction with GDP and GTP

As shown in Table I, the single substitution T25S decreases the affinity for GDP already by a factor three, due to an increase in dissociation rate, whereas the association rate is comparable to wt. The T25A substitution and the addition of substitution H22Y to T25S have more dramatic effects: dissociation rates are two orders of magnitude higher than wt, and association rates decreased. Thus the affinity constants have μM instead of nM values, a difference of 3 orders of magnitude. The fast GDP dissociation causes a technical problem concerning the evaluation of the GDP binding capacity for mutants H22Y/T25S and T25A. The half-lives of one minute and 25 s, respectively, are of the order of the filter washing time. Consequently the

loss of nucleotide during filter washing is considerable; compared to wt around 50-60% of GDP is retained on filter by the double mutant and EF-Tu(T25A) shows more variation, but still retains 25-50%. Taking this and the order of the dissociation reaction into account we estimated that the actual GDP binding capacity of these mutants should not differ much from that of EF-Tu wt (T25S proved identical). Consequently we did not apply a correction factor in evaluating experiments in the other sections. For the properties presented in this section, a standardized washing procedure reduced experimental variation. Because the calculation of the constants in this section is based on relative experimental values at different timepoints or nucleotide concentrations, this technical difficulty does not affect the values in Table I.

A related reduction of filter retention compared to the theoretical value is already observed for EF-Tu(wt)•GTP filter binding¹, and is worse for the mutants. For EF-Tu(T25A), GTP retention by the filter-bound protein was even immeasurable. For the T25S single and double substitutions, GTP dissociation rates turned out to be 3–4-fold higher than for wt. Interestingly, although for the double mutant the affinity is nearly 6-fold lower and the association rate is not much changed compared to wt, for the T25S single substitution the affinity is actually slightly higher than for EF-Tu wt, therefore the association rate is about 7-fold higher.



¹ A. Parmeggiani, unpublished observation

Activity in poly(Phe) synthesis

The most complete indication about the health of an EF-Tu mutant is its ability to support protein biosynthesis. We tested this activity by poly(U)-directed poly(Phe) synthesis at a magnesium ion concentration of 10 mM to ensure that the differences seen were not the result of insufficient saturation of EF-Tu with aa-tRNA, or insufficient binding to the ribosomal A site (see next paragraph); the GTP concentration was 400 μ M, far above the affinity values measured at 0 $^{\circ}$ C, to convert the EF-Tu to the GTP form. As shown in Fig. 1, EF-Tu(T25S) could synthesize poly(Phe) as efficiently as wt. The double mutant EF-Tu(H22Y/T25S) had a lower activity, about half that of EF-Tu wt. Surprisingly, EF-Tu(T25A), also, showed an activity that was significantly above the background level and was $\sim$ 10% that of the EF-Tu wt elongation rate.

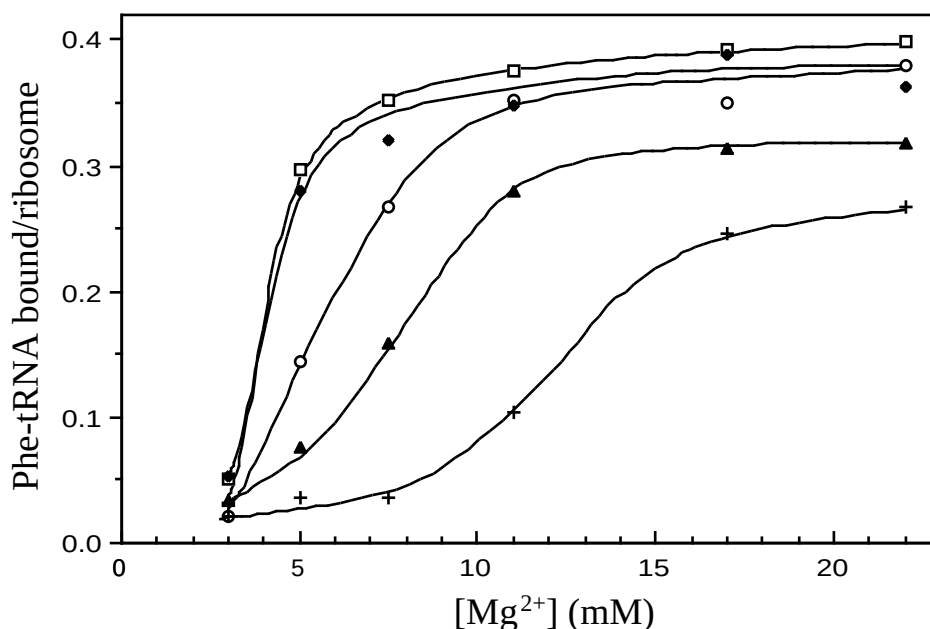


Fig. 2. **Enzymatic binding of Phe-tRNA^{Phe} to the ribosomal A site as function of [Mg²⁺].** [¹⁴C]Phe-tRNA^{Phe} bound to the ribosomal A site by EF-Tu wt (□), EF-Tu(T25A) (▲), EF-Tu(H22Y/T25S) (○) or EF-Tu(T25S) (◆) or in absence of EF-Tu (+) after a 5 min at various MgCl₂ concentrations. The reaction was started by adding together a mix containing preformed EF-Tu•GTP•[¹⁴C]Phe-tRNA^{Phe} ternary complexes (mix I) and a mix containing poly(U)-programmed ribosomes with tRNA^{Phe} preloaded in the A site (mix II). After 5 min at 30 $^{\circ}$ C the reaction (50 μ l) was filtered through nitrocellulose. Filters were immediately washed twice with 2 ml of ice-cold 50 mM TRIS-HCl, pH 7.6, 60 mM NH₄Cl and 10 mM MgCl₂, and the retained radioactivity was counted. Mix I (42 μ l) consisted of 8 μ l of a preincubated tRNA^{Phe} charging reaction and other components to give a final composition of 50 mM Hepes-KOH, pH 7.5, 70 mM NH₄Cl, MgCl₂ at variable concentration, 14 mM ME, 1.2 mM ATP, 0.24 mM GTP, 2.4 mM PEP, 30 μ g/ml pyruvate kinase, [¹⁴C]Phe-tRNA^{Phe} (formed from 0.9 μ M tRNA^{Phe} and 1.0 μ M [¹⁴C]Phe (specific activity, 1105 dpm/pmol) by 8 nM Phe-tRNA synthetase), and 0.54 μ M of the respective EF-Tu. Mix II (8 μ l) contained 50 mM Hepes-KOH, pH 7.5, 70 mM NH₄Cl, 8 mM MgCl₂, 14 mM ME, 500 μ g/ml poly(U), 1.7 μ M tRNA^{Phe} and 1.3 μ M ribosomes. Both mixes were incubated separately at 30 $^{\circ}$ C for 15 min before starting the reaction.

Enzymatic binding of aa-tRNA to programmed ribosomes

The enzymatic binding of Phe-tRNA^{Phe} to poly(U)-programmed ribosomes shows a modified dependence on Mg²⁺ concentration only for EF-Tu(T25A) and the double mutant, that of EF-Tu(T25S) being the same as that of EF-Tu wt (Fig. 2). EF-Tu(T25A) needed considerably (5-6 mM) more magnesium ion than EF-Tu wt to obtain 50% of the maximum binding of Phe-tRNA^{Phe}; moreover it was unable to reach the same plateau of binding as EF-Tu wt at any of the Mg²⁺-concentrations used, its maximum level corresponding to about 75% the control. In contrast, the double mutant displayed the same plateau level as EF-Tu wt, even though it needed approximately 3 mM more magnesium. The optimum enzymatic activity, defined as the highest ratio of enzymatic *versus* nonenzymatic binding occurred between 8 and 11 mM Mg²⁺. This prompted our choice of a standard 10 mM magnesium ion concentration for the other assays.

Ester-bond protection of aa-tRNA against spontaneous hydrolysis

The ability to participate in poly(Phe) synthesis and enzymatic binding, described in the previous section, established that the mutants have conserved either full (EF-Tu(T25S)) or at least partial activity (EF-Tu(H22Y/T25S) and EF-Tu(T25A)). On the other hand, the ability of EF-Tu to protect the ester-bond of aa-tRNA from spontaneous hydrolysis was strongly affected with the latter two mutants, as shown in Fig. 3, at 10 mM Mg²⁺. In fact EF-Tu(T25S) induced the same protection as EF-Tu wt, whereas with the other two mutants the half-life of Phe-tRNA^{Phe} decreased from several hours to approximately 20 min, *i.e.* to

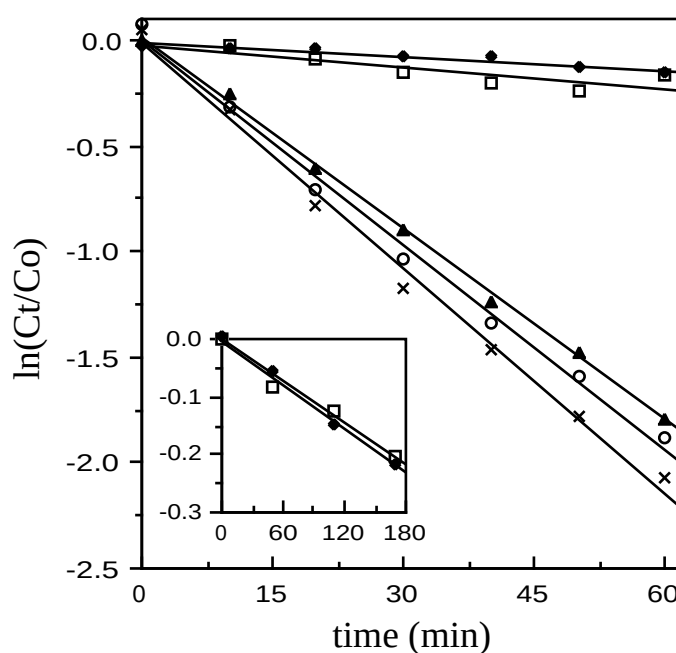


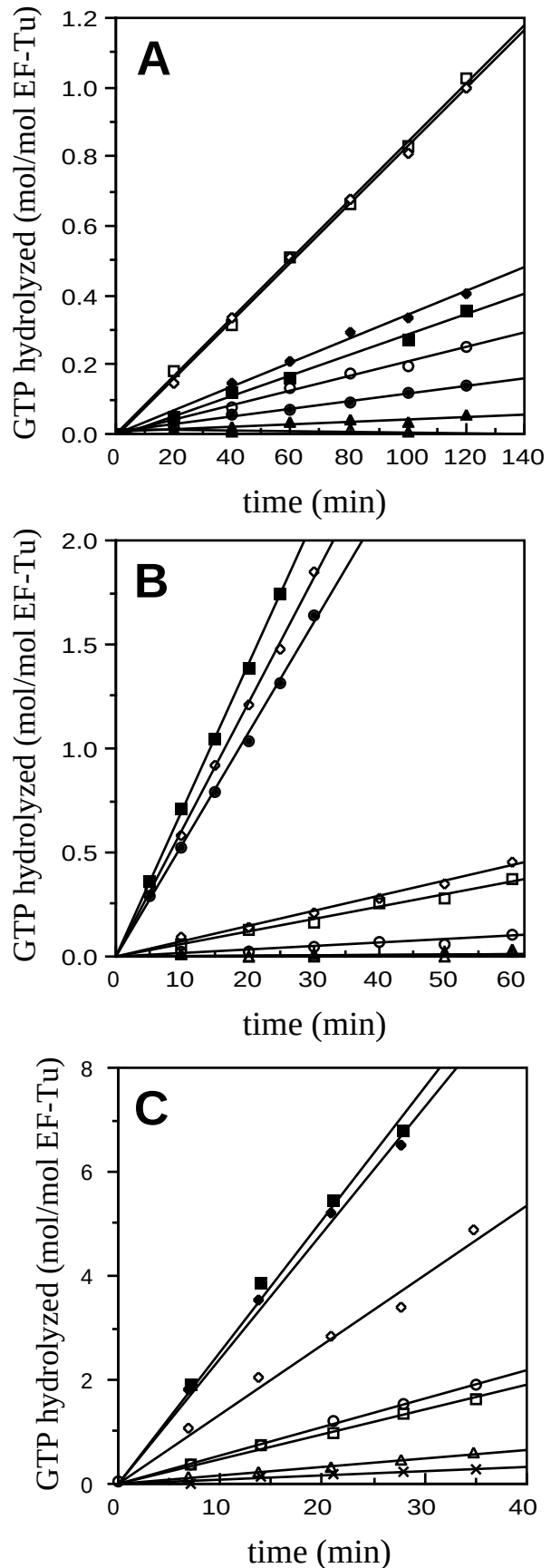
Fig. 3. **Protection against spontaneous hydrolysis of the ester bond of Phe-tRNA^{Phe} by EF-Tu.** The concentration of [¹⁴C]Phe-tRNA^{Phe} in solution was followed in time in the presence of EF-Tu wt (□), EF-Tu(T25A) (▲), EF-Tu(H22Y/T25S) (○), EF-Tu(T25S) (◆) or in absence of EF-Tu (×). The *inset* shows longer incubation times for EF-Tu wt and EF-Tu(T25S). Final reaction mixture (62 μl) contained 25 mM Hepes-KOH, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 7 mM ME, 0.2 mM GTP, 1 mM PEP, 30 μg/ml pyruvate kinase, 1.7 μM [¹⁴C]Phe-tRNA^{Phe} (specific activity, 405 dpm/pmol), and 3 μM EF-Tu as indicated. The reaction (at 30 °C) was started by adding 15 μl buffer containing the [¹⁴C]Phe-tRNA^{Phe} to 47 μl mix with the other components, which had been preincubated at 30 °C for 15 min to convert EF-Tu•GDP to EF-Tu•GTP. At the indicated times, 8-μl samples were taken, spotted onto glass fiber filters, and cold trichloroacetic acid-insoluble material on the filters was measured.

virtually the same level as obtained with Phe-tRNA^{Phe} in the absence of EF-Tu. This was surprising, especially in the case of the double mutant that was shown above to sustain a high rate of poly(Phe) synthesis and, at 10 mM Mg²⁺, nearly the same enzymatic binding of aa-tRNA to the ribosome as EF-Tu wt.

Intrinsic GTPase activity. Effect of aa-tRNA, programmed ribosomes and kirromycin

For measuring the intrinsic GTPase activity of these mutants high GTP concentrations were used to saturate the EF-Tu with nucleotide but for EF-Tu(T25A), this may

Fig. 4. GTPase activity: intrinsic and in the presence of Phe-tRNA^{Phe} (A), kirromycin (B) and ribosomes (C). Liberation of [³²P]-phosphate from [γ -³²P]GTP by EF-Tu wt ($\square$ and $\blacksquare$), EF-Tu(T25A) (Δ and $\blacktriangle$), EF-Tu(H22Y/T25S) ($\circ$ and $\bullet$), EF-Tu(T25S) ($\diamond$ and $\blacklozenge$) in the various conditions. A, in absence (*open symbols*) or presence (*filled symbols*) of 3 μ M [¹⁴C]Phe-tRNA^{Phe} (specific activity, 150 dpm/pmol). B, in absence (*open symbols*) or presence (*filled symbols*) of 50 μ M kirromycin. C, in presence of 4 μ M ribosomes, with only EF-Tu (*open symbols*) or with additionally 50 nM EF-Ts (*closed symbols*), or in absence of EF-Tu ($\times$). Final reaction conditions were 50 mM Hepes-KOH, pH 7.5, 100 mM NH₄Cl, 10 mM MgCl₂, 7 mM ME, 1 mM ATP, 50 μ M [γ -³²P]GTP (specific activity, 740-1500 cpm/pmol), 1 mM PEP, 10 μ g/ml pyruvate kinase, 2 μ M of the respective EF-Tu. EF-Tu•GTP was preformed by incubating this mix without the [γ -³²P]GTP for 10 min at 30 °C. Reactions were started after further incubation with [γ -³²P]GTP on ice for 10 min, by adding the stimulatory component and shifting the reaction to 30 °C. Appropriate-sized samples were taken at indicated times and quenched in 1 M HClO₄/3 mM KH₂PO₄, and free [³²P]-phosphate was determined by the molybdate/isopropyl acetate method (Parmeggiani & Sander, 1981).



not have been sufficient to ensure complete saturation. The intrinsic GTPase activity of the mutants *versus* that of EF-Tu wt was variously affected (Fig. 4A): for EF-Tu(T25S), it was comparable to that of EF-Tu wt and for the double mutant it was markedly reduced (~25%), whereas the rate of EF-Tu(T25A) was so low that it was difficult to determine. Interestingly, the presence of Phe-tRNA^{Phe}, which at magnesium concentrations ≤ 10 mM is known to hinder the hydrolysis of the EF-Tu wt-bound GTP (Parmeggiani & Sander, 1981; Fasano *et al.*, 1982a; Parlato *et al.*, 1983; Picone & Parmeggiani, 1983), decreased the activity of EF-Tu(T25S) by 60% as for EF-Tu wt, whereas EF-Tu(H22Y/T25S) did not always show a clear reduction, and for EF-Tu(T25A) it gave low but measurable rates with a good correlation coefficient, at ~5% of the wt intrinsic rate.

Kirromycin was able to enhance the GTPase activity of EF-Tu(T25S) and EF-Tu(H22Y/T25S), but not of EF-Tu(T25A), on which it was virtually inactive (Fig. 4B). Nevertheless, in poly(Phe) synthesis, the 50% inhibitory concentration for this mutant is even slightly lower (approximately 2 times) than for wt (not shown). Interestingly, kirromycin could compensate entirely the negative influence of the double mutation on the GTPase.

The presence of the ribosome, whether programmed with poly(U) or not (the latter not shown), markedly enhanced the GTPase activity of EF-Tu. In the absence of EF-Ts, the turnover rate of GTP hydrolysis of EF-Tu(T25S) is twice that of EF-Tu wt, as shown in Fig. 4C. This reflects the higher intrinsic GDP/GTP exchange rate of this mutant; in the presence of 50 nM EF-Ts, where the hydrolysis rate becomes limiting again, this difference disappears. The intrinsic GDP dissociation rate of the double mutant and that of EF-Tu(T25A) are sufficiently high that the turnover GTPase rate is not limited by GDP/GTP exchange (not shown). The double mutant shows the same rate as wt in the absence of EF-Ts, but if compared with wt under conditions in which the catalysis is rate-limiting (in the presence of EF-Ts), it is lower by a factor of 5. Interestingly, EF-Tu(T25A) displays a clear GTPase in the presence of ribosomes, approximately 3% of the ribosome-stimulated wt rate (when the background rate is subtracted). The turnover rates of wt and the other mutants in the presence of ribosomes are 25-35 times their intrinsic GTPase rates. That of EF-Tu(T25A) is thus stimulated by ribosome as least as much.

Dominant-negative-like properties of the two mutants

In Ras proteins, mutants of Ser-17, the residue equivalent to EF-Tu Thr-25, are known to cause a dominant negative phenotype, meaning that the mutant competes effectively with wt Ras for binding to its specific exchange factor, sequestering it and preventing activation of Ras. This was found to be associated with a strong decrease in the affinity of the mutants for GDP, and even more for GTP. To examine whether the same also holds for EF-Tu Thr-25 mutants,

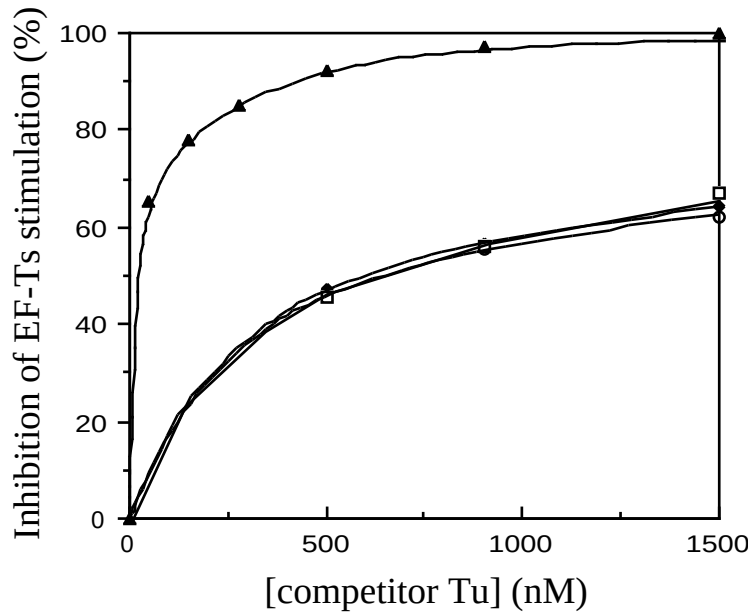


Fig. 5. **Inhibition of EF-Ts stimulation of EF-Tu(wt)•[³H]GDP dissociation by external EF-Tu•GDP.** This figure shows how the stimulation of EF-Tu(wt)•[³H]GDP dissociation by EF-Ts is inhibited by the presence of increasing concentrations of competitor EF-Tu in the (unlabelled) GDP form: EF-Tu wt (□), EF-Tu(T25A) (▲), EF-Tu(H22Y/T25S) (○), or EF-Tu(T25S) (◆). The inhibition is defined as the decrease of the ratio of the observed [³H]GDP dissociation rate (k_{obs}) and the EF-Ts-stimulated rate in the absence of competitor (k_{stim}), both corrected for the intrinsic [³H]GDP dissociation rate k_i (*i.e.* in absence of EF-Ts). Values plotted are thus $(1 - (k_{\text{obs}} - k_i)/(k_{\text{stim}} - k_i)) \times 100\%$. Reaction conditions were 50 mM Hepes-KOH, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 1 mM dithiothreitol, 30 nM EF-Tu(wt)•[³H]GDP (specific activity, 5800 dpm/pmol), 9 nM EF-Ts, 20 μM GDP, and indicated concentrations of various competitor EF-Tu•GDP. The reaction was started by adding a mix containing EF-Ts, competitor EF-Tu and excess cold GDP to the preformed EF-Tu(wt)•[³H]GDP on ice. Samples were filtered through nitrocellulose filters as for Fig. 2.

the stimulation by EF-Ts of the dissociation of radio-labeled GDP from EF-Tu wt was followed in the presence of increasing concentrations of nonlabeled EF-Tu•GDP. As shown in Fig. 5, the inhibition of the stimulation of EF-Ts on the EF-Tu(wt)•[³H]GDP dissociation as a function of the concentration of added nonlabeled EF-Tu revealed a competitive inhibitory effect—*i.e.* an effect much stronger than when the “competitor” is EF-Tu wt—only in the case of EF-Tu(T25A). This is very likely due to a sequestration of EF-Ts resulting from the formation of a stable complex with EF-Tu(T25A), as was observed also during the purification of this mutant after overexpression in *E. coli*. Interestingly, no dominant inhibitory effect could be observed with EF-Tu(T25S) and EF-Tu(H22Y/T25S), even though for the double mutant the nucleotide affinity is affected to almost the same extent as for EF-Tu(T25A).

DISCUSSION

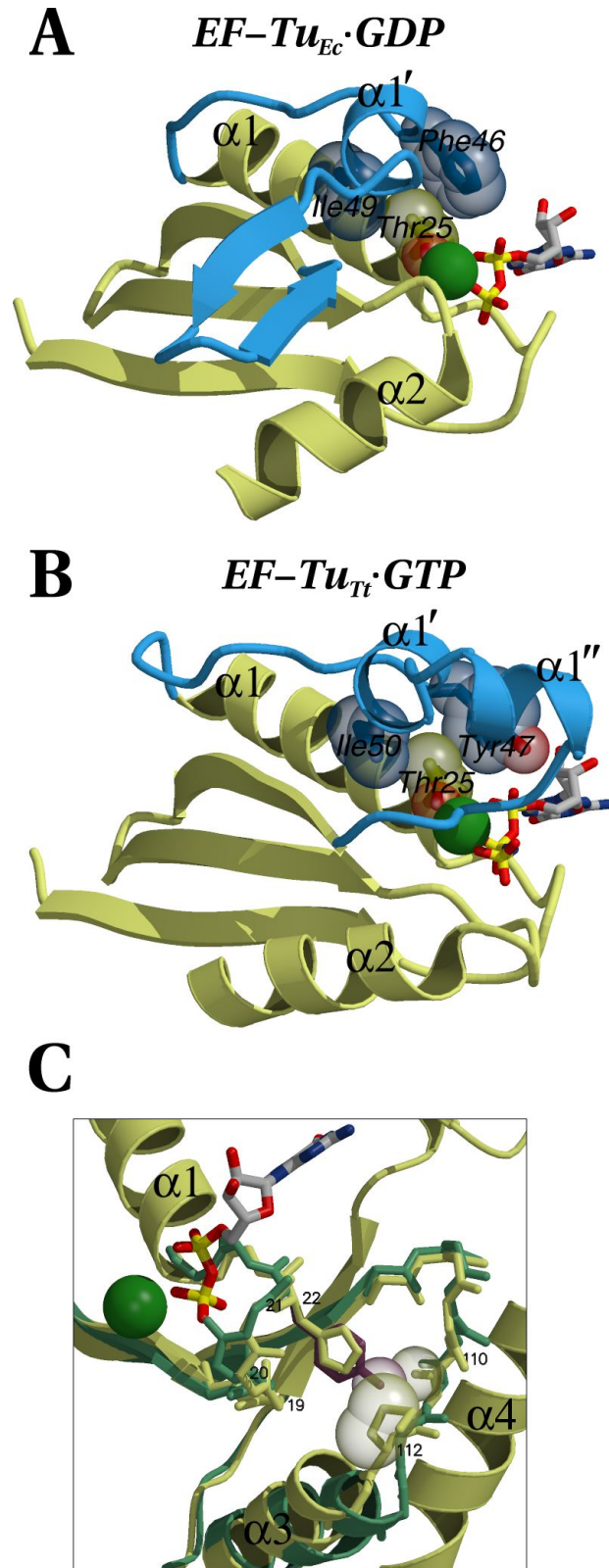
This work analyzes the effect induced on EF-Tu functions by substituting threonine 25, a strictly conserved α -helix 1 residue that flanks at the C-terminal side the P-loop (L1), and represents an essential element in the network coordinating the nucleotide-bound magnesium ion. Previous work of Hwang *et al.* (Hwang *et al.*, 1989) reported that substitution of this residue by isoleucine abolishes the interaction with the nucleotide. We decided to extend the analysis of the function of this crucial residue by introducing two other substitutions in the hope of correlating functional data with the recent impressive development of our knowledge of the three-dimensional structure of EF-Tu and its complexes. We also intended to compare the effects of this mutation with those reported for other guanine nucleotide-binding proteins on substituting the homologous residue.

The obtained results confirm the important structural role of this residue in the magnesium-substrate coordination, yet they show subtle effects not readily predictable from the current structural models. As for the nucleotide binding, the decrease in affinity of EF-Tu(T25A) for GDP by 3 orders of magnitude emphasizes the central role that the magnesium ion plays in the binding of the nucleotide to EF-Tu. EF-Tu(T25S) shows a 3-fold increase in dissociation rate, causing a subtle modification of the GDP affinity. This could be explained in terms of modification of the effector loop structure. In fact, two effector loop residues, Phe-46 and Ile-49 (Tyr-47 and Ile-50, respectively, in *Thermus thermophilus*) at both edges of the one-turn helix $\alpha 1'$ make hydrophobic contacts with the Thr-25 sidechain methyl group, forming a kind of “hydrophobic clamp” (conserved motif [Y/F]XX[I/V]) around it (Fig. 6, A and B). It represents the major interaction of this part of the effector region, in between the hypervariable first part and the conserved second part. Loss of this stabilizing hydrophobic interaction could unfavorably alter the effector loop’s structure, possibly affecting the water-mediated bond of Asp-50 with the Mg^{2+} . In eukaryal and archeal EF1 α s the conserved Thr at position 25 is replaced by a conserved Ser that lacks the β -carbon methyl group. Notably, in these factors the hypervariable part of the effector loop has in place of the hydrophobic clamp motif of prokaryal EF-Tu, a conserved very hydrophobic motif containing several bulky aromatic residues¹. This might indicate a steric compensation for the absence of the methyl group at position 25.

The surprising effect of the supplementary mutation H22Y on the nucleotide affinity of EF-Tu(T25S) is more difficult to explain. Residue His-22 is part of the P-loop pointing towards L6 preceding helix $\alpha 3$. Substitution by the approximately 1.5 Å longer tyrosine in the s orientation would cause a steric collision with the β -carbon of Met-112. Its accommodation would thus imply a displacement of L6, and consequently perhaps also of helix $\alpha 3$ (Fig. 6 C). Noteworthy, in the EF-Tu•EF-Ts complex crystal structure this helix has been displaced in the same direction by approximately 1.5 Å as a consequence of the intrusion of EF-Ts Phe-81 in

¹ I.M. Krab, unpublished observation

between EF-Tu helices $\alpha 2$ and $\alpha 3$. Thus, such a displacement might cause modifications



similar to the sequence of interactions (from His-118 via Gln-114 to the P-loop) that Wang *et al.* propose to lead to release of the nucleotide (Wang *et al.*, 1997). Another possible effect of substitution H22Y could be the deformation of the P-loop, hindering nucleotide accommodation. The slightly higher magnesium requirement for enzymatic binding indicates that some perturbation indeed occurs at the level of the magnesium binding elements in the double mutant and not with the single substitution T25S.

Fig. 6. Details of structural elements discussed in the text. A and B, close-up of the contacts between the effector region of EF-Tu, Thr-25, the nucleotide and the magnesium ion.

A, *E. coli* EF-Tu in the GDP-form (Abel *et al.*, 1996); B, *T. thermophilus* EF-Tu in the GTP form (Berchtold *et al.*, 1993). The effector region is highlighted in pale blue with residues forming the hydrophobic clamp shown as wireframe plus transparent blue Van der Waals surface, surrounding the Thr-25 side chain methyl group, indicated similarly in yellow. Magnesium (dark green) and the nucleotide are also visualized. C, close-up of the P-loop with residue His-22 and loop L6-helix $\alpha 3$. Yellow, *E. coli* EF-Tu in the GDP form (Abel *et al.*, 1996); green, structure of EF-Tu from the *E. coli* EF-Tu•EF-Ts complex (structures were aligned by least-square fitting of residues 9-35, 66-79, 97-109, 129-169, 183-199 (*E. coli* numbering)). Note the displacement of the top of helix $\alpha 3$ in the EF-Tu•EF-Ts structure relative to EF-Tu•GDP. The two loops L1 (P-loop) and L6 are represented as a backbone wireframe, with the sidechains of His-22 and Met-112 of EF-Tu•GDP shown as well. Superimposed on His-22 is a tyrosine (purple) in the same orientation. Its terminal OH group is surrounded by a transparent Van Der Waals surface, as are atoms from Ala-110 and Met-112 in loop L6 in which it would clash. Representations were made using Molscript and Raster3D (Kraulis, 1991; Merritt & Bacon, 1997).

A related observation deserves some attention. In Ras proteins mutations of the position equivalent to EF-Tu Thr-25 are known to be associated with dominant negative phenotypes (Feig & Cooper, 1988; Farnsworth & Feig, 1991; Stacey *et al.*, 1991; Créchet *et al.*, 1996). This has been shown *in vitro* for Ras2p(S24N) to be associated with increased stability of the complex of Ras2p with its exchange factor (Pouillet *et al.*, 1995; Créchet *et al.*, 1996), thus leading to sequestration of the guanine nucleotide exchange factor. Interestingly, here we describe two mutants of EF-Tu (EF-Tu(H22Y/T25S) and EF-Tu(T25A)) that have a comparable decrease in affinity for GDP, whereas only one, EF-Tu(T25A), shows dominant negative-like behaviour through increased stability of the complex with EF-Ts. Nevertheless the stimulation by EF-Ts of GDP dissociation on EF-Tu(H22Y/T25S) is not less efficient than with wt (not shown). This indicates that the interaction of the magnesium ion with Thr-25 plays a central role in the mechanism of dissociation of the EF-Tu•EF-Ts complex. In the crystal structure of the complex, the phosphate cavity is not completely open due to a peptide flip in the P-loop. If the stable interaction Mg^{2+} -Thr-25(OH) cannot be formed, the insertion of the nucleotide into the binding site and the release of EF-Ts has apparently a much lower chance of succeeding. This observation may also have implications for the reverse process, the dissociation of EF-Tu•nucleotide by EF-Ts, in which breaking of the Mg^{2+} -Thr-25(OH) bond could represent the major energetic barrier. This would emphasize the importance of the displacement of magnesium in the mechanism of stimulation of EF-Tu•GDP dissociation by EF-Ts, in agreement with the model of Kawashima *et al.* (Kawashima *et al.*, 1996).

Concerning the influence of the various mutations on the GTPase activity, the most striking observation is the different effects on the stimulation of the EF-Tu GTPase by kirromycin and ribosomes. The negative influence of the double mutation on the intrinsic GTPase can be completely compensated by kirromycin, whereas the GTPase of EF-Tu(T25A) is completely insensitive to the antibiotic, though it still interacts. In contrast, ribosomes are shown here to stimulate all mutants and wt comparably. This is probably related to a different mechanism of stimulation of the catalysis by the two agents, which are known to have an additive effect (Ivell *et al.*, 1981). The ribosome stimulation may be somehow related to that proposed for the GTPase-activating protein stimulation of small GTPases, even though some caution in comparing the two systems is in order (Krab & Parmeggiani, 1998). Our findings suggest that the enhancing effect of the ribosome is not critically dependent on the tight interaction of the magnesium ion with the core of EF-Tu, although the magnesium ion is essential for achieving the correct binding of nucleotide and the intrinsic GTPase. This is in evident contrast with the effect of the antibiotic kirromycin that we show here to stimulate the GTPase only if the Mg^{2+} -25(OH) bond is intact, thus indicating a different mechanism of action from that of the ribosome. The location and mode of binding of the antibiotic to EF-Tu are not yet precisely defined. Whereas resistance mutations were mapped to the interface of domains 1 and 3 (Abdulkarim *et al.*, 1994; Mesters *et al.*, 1994b), the known competition between EF-Ts and

kirromycin for binding to EF-Tu could rather implicate elements from the EF-Tu•EF-Ts contact area (L1(P-loop), L6- α 3, α 4 (Kawashima *et al.*, 1996)) in kirromycin binding (Krab & Parmeggiani, 1998). Consequently, kirromycin could have a direct or indirect interaction with the GTPase centre, perhaps acting through the P-loop. There it might strengthen the hydrogen bond of the Asp-21 main chain NH toward the $\beta\gamma$ -bridging oxygen, which was suggested for p21 to play a major role in the intrinsic GTPase (Maegley *et al.*, 1996).

Concerning the interaction with tRNA we have observed various effects. First, the activities in poly(Phe) and enzymatic binding indicate that EF-Tu(T25S) has conserved full ability to bind aa-tRNA, that also the double mutant at 10 mM Mg^{2+} is like wt and that even EF-Tu(T25A) has conserved at least partial activity. In surprising contrast is the complete lack of protection against spontaneous hydrolysis of the aa-tRNA ester bond by the latter two mutants. We interpret this to indicate that whereas the overall binding of aa-tRNA may not be dramatically affected, the interaction with the aminoacyl end of the tRNA is anomalous for these mutants. Interestingly, this recalls the situation observed for wt EF-Tu in the presence of the antibiotic enacyloxin IIa (Cetin *et al.*, 1996). A combination of factors probably contributes to the impaired rate of poly(Phe) synthesis with two of the three mutants: the anomalous ternary complex, the reduced efficiency of the GTPase centre, and in the case of EF-Tu(T25A) also the more stable complex with EF-Ts.

To conclude, our results underline the importance of the magnesium binding by Thr-25 in the functioning of EF-Tu, whereas on the other hand the ability of EF-Tu to participate in protein synthesis proves conserved to some extent even in the absence of this bond. This shows that modification of the magnesium-nucleotide binding network, although drastic, as in the case of substitution of the key component Thr-25 by Ala, is still compatible with an active overall conformation of the EF-Tu molecule. It also emphasizes that although the Mg^{2+} -Thr-25(OH) bond contributes greatly to the stability of nucleotide binding, other interactions are important too.

ACKNOWLEDGEMENTS

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Chapter 4

Mutagenesis of three residues, Isoleucine-60, Threonine-61 and Aspartic acid-80, implicated in the GTPase activity of *Escherichia coli* Elongation Factor Tu

Ivo M. Krab and Andrea Parmeggiani

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ABSTRACT

The properties of variants of elongation factor (EF) Tu mutated at three positions implicated in its GTPase activity are presented. Mutation I60A, which reduces one wing of a “hydrophobic barrier” screening off the nucleophilic water molecule found at the GTP γ -phosphate, causes a reduction of the intrinsic GTPase activity contrary to prediction and has practically no influence on other properties. Mutation D80N, which in the isolated G-domain of EF-Tu caused a strong stimulation of the intrinsic GTPase, reduces this activity in the intact molecule. However, whereas for wild-type EF-Tu complex formation with aa-tRNA reduces the GTPase, EF-Tu[D80N] shows a strongly increased activity when bound to Phe-tRNA. Moreover, ribosomes or kirromycin can stimulate its GTPase up to the same level as for wild-type. This indicates that a local destabilization of the magnesium binding network does not per se cause an increased GTPase but does affect its tight regulation. Interestingly, mutant D80N sequesters EF-Ts by formation of a more stable complex. Substitutions T61A and T61N induce low intrinsic GTPase, and the stimulation by ribosome is less for T61A than for T61N but still detectable, while kirromycin stimulates the GTPase of both mutants equally. This provides more evidence that stimulation by kirromycin and ribosomes follow a different mechanism. The functional implications of these mutations are discussed in the context of a transition state mechanism for catalysis. An alternative structural explanation for the strong conservation of Ile-60 is proposed.

INTRODUCTION

Elongation factor (EF) Tu is a guanine nucleotide-binding protein that takes care of the efficient transport of aminoacylated tRNAs to the A-site of the ribosome in the elongation cycle of protein biosynthesis (see Krab & Parmeggiani, 1998). It moreover plays an active role in the selection of the correct aa-tRNA from the about 40 different species available, insuring thus a high accuracy of the process. EF-Tu has specific interactions with many different partners in its functional cycle and shows remarkable structural dynamics. In the rather compact GTP form it has high affinity for aa-tRNA. The EF-Tu•GTP•aa-tRNA complex can interact with the A-site of the mRNA-programmed ribosome; in the case of correct codon-anticodon interaction this elicits a fast one-round GTP hydrolysis. The resulting GDP form has a much more open triangular structure, in which certain residues have moved through as much as 40 Å compared to the GTP form (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993; Abel *et al.*, 1996; Polekhina *et al.*, 1996), and has low affinity for aa-tRNA, thus leaving the ribosome and allowing a new peptide bond to be formed. The precise interactions changing EF-Tu from a rather inefficient GTP hydrolase into a very good one have not yet been determined. To obtain the maximum stimulation a correct codon-anticodon interaction on the ribosome is known to be an essential requirement (Swart & Parmeggiani, 1989; Rodnina & Wintermeyer, 1995; Rodnina *et al.*, 1996), although partial stimulation can be obtained by ribosomes alone (Voigt *et al.*, 1974; Parmeggiani & Sander, 1981), by high monovalent cation concentrations (Ivell *et al.*, 1981; Fasano *et al.*, 1982b) and by the antibiotic kirromycin alone (Chinali *et al.*, 1977) or even more strongly in the complex with aa-tRNA (Swart & Parmeggiani, 1989). Thus an intricate interplay seems to be necessary to obtain the optimal catalytic configuration. For the possible involvement of a residue from a ribosomal protein, or a base from rRNA, analogous to the situation for the small guanine nucleotide-binding protein family (Scheffzek *et al.*, 1998), no concrete evidence exists (Krab & Parmeggiani, 1998). Research into the residues from EF-Tu itself involved in catalysis has not been able to unequivocally attribute a catalytic role to any specific one, thus favouring a transition state stabilization mechanism for hydrolysis (Cool & Parmeggiani, 1991; Scarano *et al.*, 1995; Krab & Parmeggiani, 1998).

Among the residues conserved in the elongation factors are those involved in nucleotide binding [four groups of residues forming the guanine nucleotide-binding protein “fingerprint” ([Bourne, 1991 #4; Kjeldgaard, 1996 #23])], and also others that are characteristic for this guanine nucleotide-binding protein family but whose function is not clear. In the first group are Thr-61 and Asp-80, two residues having a direct and a water-mediated interaction with the essential magnesium complexed to the nucleotide, respectively. In the second group is Ile-60, a residue that was found in the 3D GTP structure to form a “hydrophobic barrier”, together with Val-20 located opposite to it in the P-loop. This “barrier” would screen off the water molecule located at the γ -phosphate in position for a nucleophilic attack. In this context, His-84 is

considered the general base activating this water molecule, which supposes that the “gate” opens periodically by thermal movement, allowing the catalysis by His-84 to take place.

In this work we set out to define more precisely the role of the magnesium ion and the hydrophobic barrier in the GTPase reaction by characterizing mutants of these three residues. In the isolated domain 1 (G-domain) the substitution Asp-80→Asn was found to cause a strong increase in the intrinsic GTPase rate, concomitant with a structural destabilization (Harmark *et al.*, 1992). This effect suggested a destabilization of the magnesium binding network as an intermediary step of the transition leading to the hydrolysis of GTP. We extended that work here by examining the same mutation in the intact EF-Tu. Thr-61 was suggested to be involved in transmitting the ribosome signal for triggering the GTPase on the basis of mutations of the homologous residue in *Thermus thermophilus* EF-Tu (Ahmadian *et al.*, 1995). We present an in-depth analysis of mutants of Thr-61 to Ala and Asn, in which differences between the *T. thermophilus* and *Escherichia coli* situations are revealed. To evaluate the proposed role of the “hydrophobic gate” residue Ile-60 in reducing the intrinsic GTPase, we characterized the mutation Ile-60→Ala, which complements previous work describing the properties of Val-20→Gly (Jacquet & Parmeggiani, 1988).

EXPERIMENTAL PROCEDURES

Biological materials

Ribosomes, EF-G, Phe-tRNA^{Phe} synthetase, and partially purified tRNA^{Phe} (5 to 70%) were prepared from *E. coli* as reported (Chinali *et al.*, 1977; Parmeggiani & Sander, 1981). In the case of tRNA^{Phe} also purified preparations obtained from Sigma were used.

Construction, overproduction and purification of EF-Tu mutants

For rapid purification purposes wt EF-Tu and mutants were overproduced as fusion with glutathione S-transferase and purified as described before (Krab & Parmeggiani, 1999a). Mutations were introduced by the “Unique Site Elimination” method (Deng & Nickoloff, 1992), using a kit from Pharmacia. As a substrate for the mutagenesis reactions we used pGtAM, a derivative of plasmid pGEX-2T*tufA* (Anborgh, 1993), which codes for a thrombin-cleavable glutathione S-transferase-fused *E. coli* EF-Tu (*tufA*). In pGtAM the NcoI site from pGEX-2T*tufA* has been eliminated, and the unique NruI site in the *tufA* sequence—which turned out to digest poorly—has been modified by a silent mutation generating a unique overlapping NcoI site, used for the selection procedure. The mutagenic oligonucleotides for the amino acid substitutions were 5'-CT CGT GGT GCC ACC ATC AA -3' for I60A, 5'-GT GGT ATC GCC ATC AAC-3' for T61A, 5'-CGT GGT ATC AAC ATC AAC AC-3' for T61N, and 5'-CAC GTA AAC TGC CCG-3' for D80N (modified bases underlined). Because

transformation by the calcium chloride method advised in the kit proved not efficient enough, the “simple and efficient method” of Inoue was used instead (Inoue *et al.*, 1990). Selected clones were verified by ds-DNA sequencing of the plasmid, and a mutagenesis efficiency of more than 50% was found. The concentration of the purified proteins was determined by the Bio-Rad protein assay, using bovine serum albumin as standard.

Functional assays

Poly(Phe) synthesis was determined kinetically from the formation of hot trichloroacetic acid-insoluble material (Swart *et al.*, 1987) and the GTPase activity from liberation of [³²P]P_i using the molybdate method (Parmeggiani *et al.*, 1971). Nucleotide dissociation rates and affinities (Ivell *et al.*, 1981; Anborgh & Parmeggiani, 1991; Cool & Parmeggiani, 1991) and protection of aa-tRNA by EF-Tu against spontaneous hydrolysis (Anborgh & Parmeggiani, 1993) were measured as described. Detailed reaction conditions are reported in the legends to the figures.

RESULTS

Production, expression and purification of mutant EF-Tu's

EF-Tu wt and mutants I60A, T61A and T61N are overexpressed to a high level and show good solubility (>70%),

giving yields of up to 2 mg EF-Tu (unfused)/g of cells (wet weight). Mutant D80N, on the other hand, is well expressed but less of the protein is found in the S30 supernatant fraction. This mutant also shows a high stability of the complex with EF-Ts, making an ultimate Superdex 75

Table 1: Nucleotide interaction parameters^a

	GDP			GTP		
	$k_{\text{off}} \times 10^4$ (s ⁻¹)	$k_{\text{on}} \times 10^{-4}$ ^b (s ⁻¹ M ⁻¹)	K_d (μM)	$k_{\text{off}} \times 10^4$ (s ⁻¹)	$k_{\text{on}} \times 10^{-4}$ ^b (s ⁻¹ M ⁻¹)	K_d (μM)
wt	2.2	34	0.00064	79	2.3	0.34
I60A	2.1	34	0.00062	163	1.5	1.1
T61A	2.6	43	0.00061	165	4.1	0.40
T61N	2.3	38	0.00060	187	2.9	0.64
D80N	260	1.9	1.4	128	~0.3 ^c	~5 ^c

^a Shown are apparent rate constants for dissociation (k_{off}) and association (k_{on}) of GDP and GTP and the equilibrium dissociation constants (K_d) measured at 0 °C. ^b Calculated as $k_{\text{on}} = k_{\text{off}}/K_d$ assuming a two-state association/dissociation reaction without intermediate steps. ^c Value estimated from nucleotide binding versus nucleotide concentration.

gel filtration step necessary to separate free protein from the EF-Tu[D80N]•EF-Ts complex in order to obtain an EF-Ts-free preparation. These observations agree with our recent findings (unpublished) that several EF-Tu mutants with lower nucleotide affinity have problems to fold correctly in the cell. It does not indicate any structural instability, however. No precipitation phenomena or other stability problems were observed with the purified factors.

Interaction with GDP and GTP

Concerning the dissociation constants and the dissociation rate constants, we found that for the GDP interaction the mutants of the effector region residues 60 and 61 have parameters that are not far from those of wt EF-Tu (Table 1). For the GTP interaction we see an increase in the dissociation rates by a factor of about 2, whereas the affinity is lower by about a factor 3 for EF-Tu[I60A], and by a factor 2 for EF-Tu[T61N].

The mutant of residue 80, on the other hand, shows a dramatic alteration. The GDP affinity is 3 orders of magnitude lower, the dissociation rate being 2 orders of magnitude higher. As a consequence, the deduced association rate is also more than 1 order of magnitude lower. For GTP the changes are less dramatic. The affinity is again lower than for wt, but the difference is only 1 order of magnitude and the dissociation rate is less increased than for the other mutants.

Activity in poly(Phe) synthesis

To evaluate the overall effect of the mutations on EF-Tu's ability to sustain its physiological function, we performed poly(U)-directed poly(Phe) synthesis (Figure 1) at a magnesium concentration of 10 mM to ensure that the differences seen are not the result of insufficient saturation of EF-Tu•GTP with aa-tRNA (see below). We observed that EF-Tu[I60A] can sustain the same rate as wt, and that EF-Tu[T61N] is little affected, showing 80% of the wt rate. On the other hand, EF-Tu[T61A] and EF-Tu[D80N] are less efficient, at 28% and 25%, respectively. They are thus almost equally affected in their physiological function, despite the quite different effects of the mutation on the nucleotide interaction. The two different mutations of Thr-61 behave also strikingly

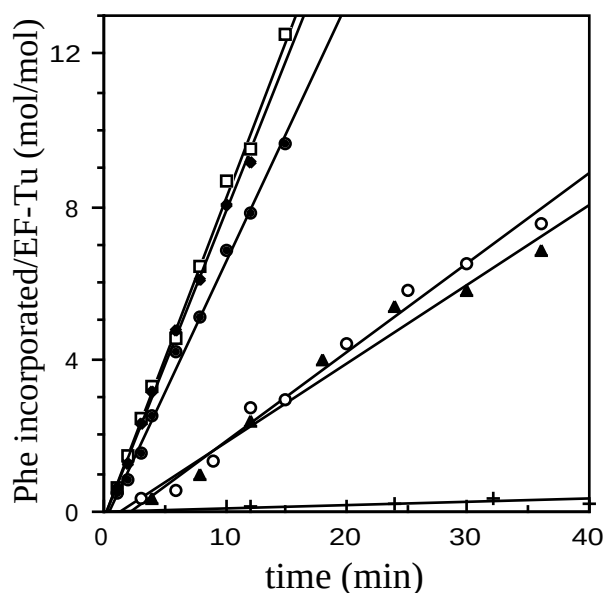


FIGURE 1: Poly(U)-directed poly(Phe) synthesis by EF-Tu wt ($\square$), EF-Tu[I60A] ($\blacklozenge$), EF-Tu[T61A] ($\circ$), EF-Tu[T61N] ($\bullet$), and EF-Tu[D80N] ($\blacktriangle$) or in absence of EF-Tu (+). The final reaction mixture (60 μ L) contained 50 mM Hepes-KOH, pH 7.5, 70 mM NH_4Cl , 10 mM MgCl_2 , and 7 mM ME (buffer A) with 0.9 mM ATP, 1.0 mM GTP, 3.8 mM phosphoenolpyruvate, 30 $\mu\text{g/mL}$ pyruvate kinase (EC 2.7. units/mL myokinase (EC 2.7.4.3), 50 nM EF-Ts, 0.8 μM EF-G, 20 μM [^{14}C]Phe (specific activity 147 dpm/pmol), 3 μM tRNA^{Phe}, 26 nM phenylalanine-tRNA synthetase (EC 6.1.1.20), 1.5 μM ribosomes with tRNA^{Phe} preloaded in the P-site, 100 $\mu\text{g/mL}$ poly(U), and 0.5 μM EF-Tu as indicated. The reaction was started by adding together a mix containing ribosomes, poly(U), and tRNA^{Phe} in buffer A and a mix containing all other factors and components which had been separately incubated at 30 $^\circ\text{C}$ for 15 min. Incubation was continued at 30 $^\circ\text{C}$, 6 μL samples were withdrawn at indicated times and spotted on glass fiber filters, and hot trichloroacetic acid-insoluble radioactivity was determined.

differently, pointing to the importance of the Thr-61-OH→Mg²⁺ bond for the dynamic cycle.

Enzymatic binding of aa-tRNA to programmed ribosomes

To analyze the effect of the mutations on the Mg²⁺ requirement for function, we examined the extent of enzymatic binding of Phe-tRNA^{Phe} to the A-site of programmed ribosomes as a function of the Mg²⁺ concentration (Figure 2). EF-Tu[I60A] is indistinguishable from wt, as could be expected from the absence of an interaction with the magnesium. EF-Tu[T61N] and EF-Tu[D80N] both reach 50% of the maximum binding at 1.5-2 mM higher [Mg²⁺], whereas EF-Tu[T61A] needs about 2.5 mM more than wt to reach this level. These results show a close qualitative agreement with the expected ability of the substituting residue to still complex the magnesium ion. They also show that the residue at position 61 does not really need to have an interaction with the magnesium ion for EF-Tu to be able to sustain this partial reaction efficiently, whereas in the complete elongation cycle loss of this contact leads to a much reduced activity.

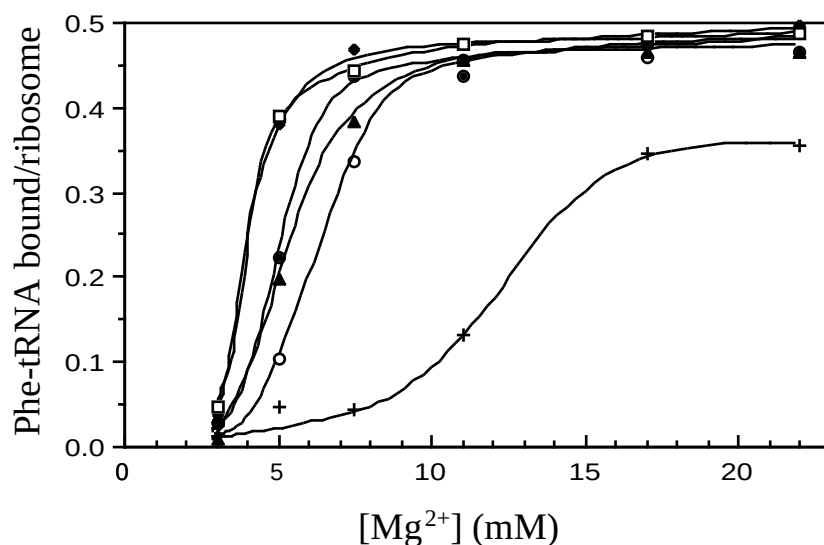


FIGURE 2: Enzymatic binding of [¹⁴C]Phe-tRNA^{Phe} to the ribosomal A site as function of [Mg²⁺] by EF-Tu wt (□), EF-Tu[I60A] (◆), EF-Tu[T61A] (○), EF-Tu[T61N] (●), and EF-Tu[D80N] (▲) or in absence of EF-Tu (+). The reaction was started by adding together a mix containing preformed EF-Tu•GTP•[¹⁴C]Phe-tRNA^{Phe} ternary complexes (mix I) and a mix containing poly(U)-programmed ribosomes with tRNA^{Phe} preloaded in the A-site (mix II). After 5 min at 30 °C the reaction (50 µL) was filtered through nitrocellulose. Filters were immediately washed twice with 2 mL of ice-cold buffer, and the retained radioactivity was counted. Mix I (42 µL) consisted of 8 µL of a preincubated tRNA^{Phe} charging reaction and other components to give a final composition of 50 mM Hepes-KOH, pH 7.5, 70 mM NH₄Cl, MgCl₂ at variable concentrations, 14 mM ME, 1.2 mM ATP, 0.24 mM GTP, 2.4 mM phosphoenolpyruvate, 30 µg/mL pyruvate kinase, [¹⁴C]Phe-tRNA^{Phe} [formed from 0.9 µM tRNA^{Phe} and 1.0 µM [¹⁴C]Phe (specific activity 1105 dpm/pmol) by 8 nM phenylalanine-tRNA synthetase], and 0.54 µM each EF-Tu. Mix II (8 µL) contained 50 mM Hepes-KOH, pH 7.5, 70 mM NH₄Cl, 8 mM MgCl₂, 14 mM ME, 500 µg/mL poly(U), 1.7 µM tRNA^{Phe}, and 1.3 µM ribosomes. Both mixes were incubated separately at 30 °C for 15 min before the reaction was started.

Ester bond protection of aa-tRNA

Whereas the ability to participate in poly(Phe) synthesis and enzymatic binding, shown in the previous experiments, establishes that the mutants have conserved the ability to productively interact with aa-tRNA, we also tested whether the property of EF-Tu to protect the ester bond of aa-tRNA from spontaneous hydrolysis has been altered by the mutations. As shown in Figure 3, at 10 mM Mg^{2+} marked differences become evident. In fact, whereas the behaviour of EF-Tu[I60A] is again undistinguishable from wt, both mutations of the adjacent Thr-61 are equally less effective: the half-life of aa-tRNA goes down from >10 h to 90 min. Mutant EF-Tu[D80N] protects slightly better than the Thr-61 mutants. Since the affinities for aa-tRNAs are in the nanomolar range (Abrahamson *et al.*, 1985; Louie & Jurnak, 1985), at the μM concentrations used all mutant EF-Tu should at least be near-saturated with aa-tRNA. Thus, the reduced aa-esterbond protection for both Thr-61 mutants and for EF-Tu[D80N] points to an anomalous interaction of the aminoacyl end of aa-tRNA with EF-Tu, whereas the orientation of the other EF-Tu domains to aa-tRNA seems to be little or not affected.

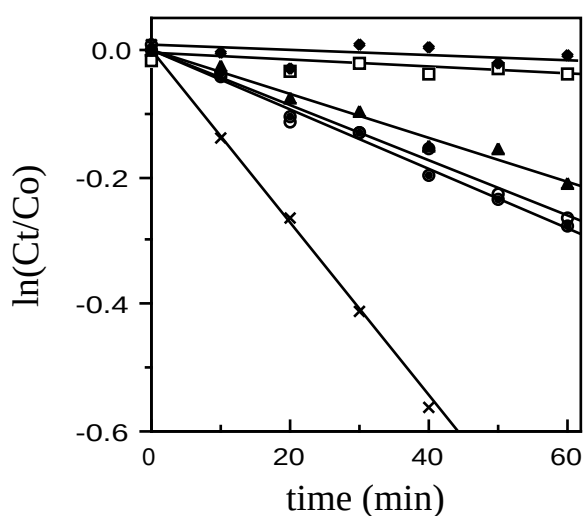


FIGURE 3: Protection against spontaneous hydrolysis of the ester bond of Phe-tRNA^{Phe} by EF-Tu wt (□), EF-Tu[I60A] (◆), EF-Tu[T61A] (○), EF-Tu[T61N] (●), and EF-Tu[D80N] (▲) or in absence of EF-Tu (×). The final reaction mixture (62 μL) contained 25 mM Hepes-KOH, pH 7.5, 60 mM NH_4Cl , 10 mM $MgCl_2$, 7 mM ME, 0.5 mM GTP, 1 mM phosphoenolpyruvate, 30 $\mu g/mL$ pyruvate kinase, 1.5 μM [^{14}C]Phe-tRNA^{Phe} (specific activity 331 dpm/pmol) and 3 μM EF-Tu as indicated. The reaction (at 30 °C) was started by adding 15 μL buffer containing the [^{14}C]Phe-tRNA^{Phe} to 47 μL of mix with the other components which had been preincubated at 30 °C for 15 min to convert EF-Tu•GDP to EF-Tu•GTP. At indicated times 8 μL samples were taken and spotted onto glass fibre filters, and cold-trichloroacetic acid-insoluble material on the filters was measured.

Intrinsic GTPase activity. Effect of aa-tRNA, ribosomes and kirromycin

Our main interest in characterizing these mutant EF-Tu's concerned the effects on the GTPase, both intrinsic and in interaction with EF-Tu's physiological partners. Table 2 summarizes the activities measured starting from EF-Tu• $[\gamma\text{-}^{32}P]GTP$, at high $[\gamma\text{-}^{32}P]GTP$ concentration (100 μM), which should ensure near-complete saturation even for EF-Tu[D80N], so that the measured rates correspond closely to V_{max} . The wild-type shows the usual reduction in the complex with Phe-tRNA, by a factor of 5 in these circumstances. Empty ribosomes stimulate the GTPase 42 times. For the substitution I60A, the hydrophobic barrier hypothesis predicts an increased intrinsic GTPase rate. On the contrary, we observe a decrease by 35%.

Table 2: GTPase activities^a

	intrinsic $k_{app} \times 10^5 \text{ (s}^{-1}\text{)}$	+Phe-RNA ^{Phe} $k_{app} \times 10^5 \text{ (s}^{-1}\text{)}$	+ribosomes $k_{app} \times 10^5 \text{ (s}^{-1}\text{)}$
wt	8.0	1.6	334
I60A	5.2	2.9	178
T61A	1.4	2.0	8.8
T61N	1.6	2.4	50.9
D80N	3.7	19.2	326

^a Apparent rate constants of the liberation of [³²P]-phosphate from [γ -³²P]GTP at 30 °C. Final reaction conditions were 50 mM Hepes-KOH, pH 7.5, 100 mM NH₄Cl, 10 mM MgCl₂, 7 mM ME, 1-2 mM ATP, 100 μ M [γ -³²P]GTP (specific activity 1500 cpm/pmol), 1 mM phosphoenolpyruvate, 10 μ g/mL pyruvate kinase, and 3-4 μ M each EF-Tu. EF-Tu•GTP was preformed by incubating this mix without the [γ -³²P]GTP for 15 min at 30 °C. Reactions were started after further incubation with [γ -³²P]GTP on ice for 15 min by adding the stimulatory component and shifting the reaction to 30 °C. Samples were taken at indicated times and quenched in 1 M HClO₄/3 mM KH₂PO₄, and free [³²P]-phosphate was determined by the molybdate/isopropyl acetate method (Parmeggiani & Sander, 1981). The background hydrolysis by ribosomes subtracted in the third column was 15.9 s⁻¹.

Complexation with Phe-tRNA^{Phe} further reduces the GTPase, although it stabilizes this mutant less than wt. Ribosomes stimulate this mutant quite well, though at 34 times also somewhat less efficiently than wt. Both mutants of the neighbouring residue Thr-61 show a ca. 5-fold reduced intrinsic GTPase, and in contrast to wt, the presence of Phe-tRNA^{Phe} slightly stimulates their activity. For these mutants the ability of ribosomes to stimulate the GTPase varies with the substitution. Whereas the EF-Tu[T61N] is stimulated by ribosomes 32 times, T61A's rate is enhanced only six times. The most surprising behaviour is shown by EF-Tu[D80N], whose intrinsic activity is decreased to about half. However, in the presence of Phe-tRNA^{Phe} the GTPase rate is increased to about 2.5 times the wt intrinsic rate, which corresponds to a 5-fold stimulation compared to this mutant's intrinsic catalytic activity. We furthermore observe that EF-Tu[D80N] is stimulated to the same rate as wt in the presence of ribosomes, ie. by a factor of 88.

To verify that the surprising decreased GTPase for EF-Tu[I60A] is due to a decrease in the catalytic efficiency and not some other effect, we determined both K_M and k_{cat} from a Lineweaver-Burke plot (not shown). The k_{cat} is decreased from $7.1 \cdot 10^{-5} \text{ s}^{-1}$ in the wt to $4.1 \cdot 10^{-5} \text{ s}^{-1}$ in EF-Tu[I60A], whereas the K_M is slightly increased from 0.26 to 0.44 μ M. Thus the catalytic efficiency k_{cat}/K_M is reduced from 270 to 93 M⁻¹ s⁻¹ for this mutant.

Concerning the GTPase in the presence of the antibiotic kirromycin, we observe for EF-Tu[D80N] the same stimulated rate as wt, similar to the activation by ribosomes; for both mutants of Thr-61 we see a moderate enhancement of about 5-fold, whereas EF-Tu[I60A] is stimulated comparably to wild-type (results not shown).

Dominant-negative-like properties of the mutants

We recently reported that EF-Tu[T25A] shows a behaviour similar to that of so-called dominant-negative mutants of Ras proteins, i.e., mutants that inhibit native Ras in the cell by sequestration of the exchange factors that regenerate the active form of Ras. Such mutations causing increased stability of the complex with the exchange factor occur in Ras proteins in position 17 (the equivalent of EF-Tu Thr-25) but also in position 57, the equivalent of EF-Tu Asp-80 (Jung *et al.*, 1994). Interestingly, during purification of EF-Tu[D80N] we again observed tight complex formation with EF-Ts, as for EF-Tu[T25A]. Therefore, we tested whether EF-Tu[D80N] could also inhibit the stimulation by EF-Ts of [^{3} H]GDP exchange on wt EF-Tu. As shown in Figure 4, the presence of increasing concentrations of mutant strongly inhibits the function of EF-Ts. The other mutants do not show this effect (not shown). The observed differences in behaviour between EF-Tu[T25A] and a double mutant EF-Tu[H22Y/T25S] had led us to conclude that this phenomenon is related to the ability of EF-Tu to have a stable interaction with the magnesium ion from position 25 and not simply to the strong decrease in nucleotide affinity observed for these two mutants. Thus, we may assume that also for EF-Tu[D80N] the changed nature of the interaction with the Mg^{2+} •nucleotide is responsible for the increased stability of the EF-Tu•EF-Ts complex. This once again confirms the importance of the magnesium ion in the pathway for nucleotide ejection by EF-Ts.

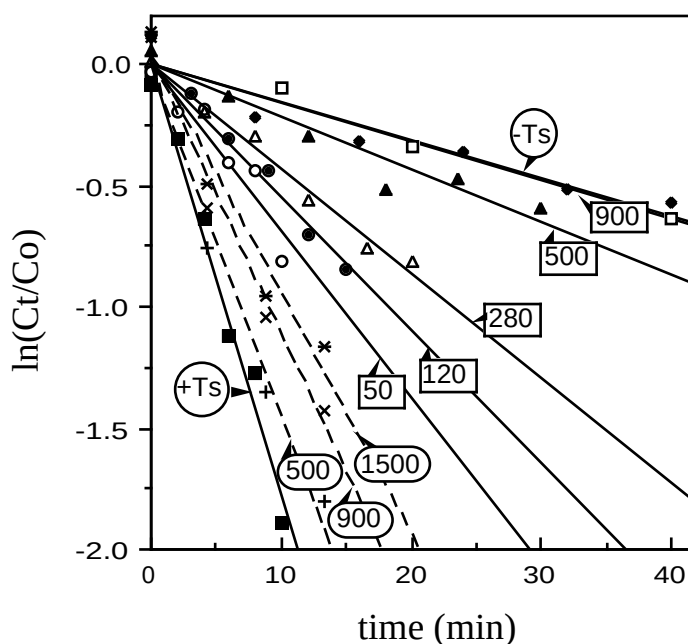


FIGURE 4: Inhibition of EF-Ts stimulation of EF-Tu[wt]•[^{3} H]GDP dissociation by increasing concentrations of external EF-Tu[wt]•GDP (dotted lines and oval labels) or EF-Tu[D80N]•GDP (solid lines and square labels). Numbers in the labels indicate the concentration of competing EF-Tu (in nM) in the reaction for the corresponding curve. The curves labelled +Ts and -Ts are the control reactions without competitor in the presence and absence, respectively, of EF-Ts. Reaction conditions were 50 mM Hepes-KOH, pH 7.5, 60 mM NH_4Cl , 10 mM $MgCl_2$, 1 mM dithiothreitol, 30 nM EF-Tu[wt]•[^{3} H]GDP (specific activity 6300 dpm/pmol), 9 nM EF-Ts, 20 μ M GDP, and indicated concentrations of various competitor EF-Tu•GDP. The reaction was started by adding a mix containing EF-Ts, competitor EF-Tu, and excess cold GDP to the preformed EF-Tu[wt]•[^{3} H]GDP on ice. Samples were filtered through nitrocellulose filters as for Figure 2.

DISCUSSION

This work analyzes the effect of mutations of three conserved residues of EF-Tu from *E. coli*, two of which (Thr-61 and Asp-80) are components of the magnesium binding network (illustrated in Figure 5A) and one (Ile-60) is a close neighbour to a water molecule located at the γ -phosphate of GTP (Figure 5B). Residues Ile-60 and Thr-61 are part of the switch I region of EF-Tu. In the GTP form they flank at the C-terminal side the short α helix $\alpha 1''$, which in the GDP form unwinds to a β -hairpin bridging helix $\alpha 2$ and domain 3 (Abel *et al.*, 1996; Polekhina *et al.*, 1996) (Figure 5C). The third residue examined, Asp-80, is strictly conserved in the DXXG motif just preceding the switch II region of guanine nucleotide-binding proteins. Residues Thr-61 and Asp-80 interact directly and via a water molecule, respectively, to the nucleotide-bound magnesium ion which is essential for EF-Tu's function (Miller & Weissbach, 1977). All three residues were implicated in the GTPase reaction in various ways in the literature. Most notably, substitution D80N increased the intrinsic GTPase in the isolated domain 1 of EF-Tu by up to 2 orders of magnitude, although the protein was strongly destabilized and high concentrations of glycerol were necessary to preserve its activity (Harmark *et al.*, 1992). Upon substitution of the homologue of Thr-61 to Ala, *T. thermophilus* EF-Tu was reported to have very low intrinsic GTPase activity, which could not be stimulated by ribosomes; thus this residue was suggested to play a role in transmitting the “ribosome signal” for triggering the GTPase (Ahmadian *et al.*, 1995). Ile-60, finally, was found in the GMPPNP crystal structure to be part of a hydrophobic barrier between the putative nucleophilic water located at the γ -phosphate of the nucleotide and residue His-84, which had been proposed to act as a general base in the catalysis of GTP hydrolysis (Berchtold *et al.*, 1993). Thus, it was predicted that removing or reducing this barrier would facilitate access of the outward-pointing His-84 side chain into the active site and therefore increase the GTPase activity of the protein.

Concerning the effect on the nucleotide interaction, the mutants can be divided into two groups: mutations of position 60 and 61 do not influence the interaction with GDP and very modestly affect the interaction with GTP; most notably they show a slightly faster GTP dissociation. Since EF-Tu[I60A] has no direct contacts with either nucleotide or magnesium, a local conformation disturbance seems the most likely explanation for its higher GTP dissociation rate. The similar and remarkably modest effect of the Thr-61→Ala mutation might seem to in contrast with the prominence of the Thr-61–Mg²⁺ ion link in the crystal structures, also when compared to the drastic change caused by mutation of the opposite Thr-25 to alanine (Krab & Parmeggiani, 1999a). However, this finding agrees with experimental evidence that in H-ras p21 the coordination of the homologous residue Thr-35 to magnesium is rather weak (Halkides *et al.*, 1994; Farrar *et al.*, 1997), despite the presence of a similarly prominent interaction in the crystal. In EF-Tu the coordination may be even weaker, for the ras mutation T35A shows an

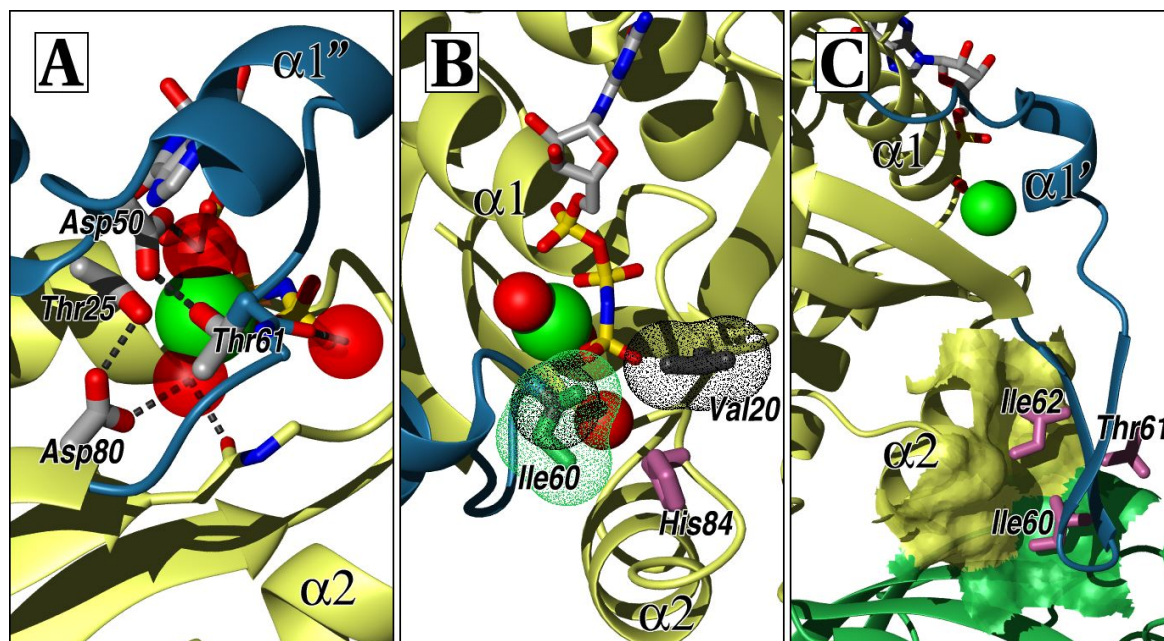


FIGURE 5: Details of structural elements discussed in the text. Residue numbers refer to *E. coli* even if the structure is from *T. thermophilus*. Panel A: Detailed view of the residues surrounding the Mg^{2+} ion (green ball) in the EF-Tu_{Tt}•GTP structure (Berchtold *et al.*, 1993) and their interactions with each other and the water molecules (red transparent balls). Dotted lines indicate hydrogen bonds (distances 2.6-2.8 Å). The effector region (switch 1) is colored blue; the nucleotide is in part covered by the magnesium ion, water molecules, and helix $\alpha 1''$ from the effector region. Panel B: Close-up of the hydrophobic barrier (EF-Tu_{Tt}•GTP), in the wt situation and with an Ala statically modeled in place of Ile-60. His-84 (purple) is prevented from approaching the water molecule at the γ -phosphate by residues Val-20 and Ile-60, while an Ala at position 60 leaves more space for the barrier to open through thermal motion, assuming no dramatic changes of the local conformation. Van der Waals envelopes of these residues are indicated in black dots for the modeled situation in the mutant, while the wt Ile-60 surface is dotted in green. Panel C: Overview of the structure of the switch 1 region (blue) in EF-Tu_{Ec}•GDP (Abel *et al.*, 1996) and the hydrophobic contacts of residues Ile-60 and Ile-62 (purple) with the interface of helix $\alpha 2$ and domain 3 (green), indicated as a partially transparent solvent-accessible contact surface below the switch 1 β -hairpin. Ile-62 is in a hydrophobic pocket formed by Ala-85, Lys-89 and Asn-90 (not shown), whereas Ile-60 is surrounded by Lys-89, Thr-93, Tyr-309 and Ala-385 (not shown). Thr-61 located between the isoleucines points into the solvent, far away from the magnesium ion (green ball). Figures prepared using MOLMOL (Koradi *et al.*, 1996) and POV-ray (<http://www.povray.org>).

approximately 6-fold decreased affinity for GTP, whereas the affinity of EF-Tu[T61A] practically equals that of wt. Noteworthy, the influence on the dissociation rates shown here is opposite to that found for EF-Tu_{Tt}: there, substitutions T62A and T62S increased the GDP dissociation rates but did not affect the GTP dissociation (Ahmadian *et al.*, 1995).

On the other hand, the substitution Asp-80→Asn shows a dramatic change of all interaction parameters. Most notably, the GDP affinity lies in the micromolar range, i.e., close to that for GTP. Thus, it mostly loses the several orders of magnitude higher affinity for GDP, typical for *E. coli* EF-Tu. Aspartate has a pK_a of 4.4 and is thus mostly ionized, making it an exclusiv hydrogen bond acceptor. In the crystal structures Asp-80 is seen to accept hydrogen bonds from Thr-25 and from one water coordinated to the magnesium, which donates its other H

towards the Cys-82 mc CO group. An asparagine side-chain is poorly ionizeable and can act both as a hydrogen bond acceptor and as a donor. Whereas a similar configuration as for Asp-80 is possible with the Asn amide donating an H to the Mg^{2+} -bound water, leaving one amide and one water H unbonded, the lack of a negative charge attracting the magnesium likely makes the bonding weaker.

Concerning the effects on the GTPase, EF-Tu[I60A] is shown here to have a decreased intrinsic GTP hydrolysis, in contrast to the prediction that reducing the hydrophobic barrier should increase the intrinsic catalysis rate. This finding is similar to that obtained several years ago with EF-Tu[V20G], removing the other wing of the hydrophobic gate, which displayed a reduced GTPase (4–5-fold) (Jacquet & Parmeggiani, 1988). Differently, however, whereas ribosomes hardly stimulated the EF-Tu[V20G] GTPase, EF-Tu[I60A] is stimulated to a similar extent as wt, as is the case for kirromycin. Thus, the modification seems to affect the stimulated GTPase in a similar way as the intrinsic reaction, suggesting that the effect is unspecific and that the residue is not directly involved in the mechanism of catalysis. Also mutagenesis of the proposed catalytic residue His-84 had shed doubt on its involvement in water activation, at least for the intrinsic reaction (Cool & Parmeggiani, 1991; Scarano *et al.*, 1995; Zeidler *et al.*, 1995), and comparison with the various recent crystal structures of Ras(-like) proteins with their respective GTPase-activating proteins in a transition state-mimicking complex (Rittinger *et al.*, 1997b; Scheffzek *et al.*, 1997; Nassar *et al.*, 1998) also raises questions considering the similarity of the GTPase stimulation in the two systems (Krab & Parmeggiani, 1998). An important still unsolved question remains then, why Ile-60 is so highly conserved in elongation factors (conservatively substituted to Val in most archaea). A possible answer may be found in the 3D structure of the GDP form of EF-Tu (Figure 5C): The hairpin loop resulting from the unwinding of helix $\alpha 1$ forms a bridge over helix $\alpha 2$ and domain 3. Both Ile-60 and the universally conserved Ile-62 are located in hydrophobic pockets in the interface between the loop and the other two elements, whereas Thr-61 in between these two residues points into the solvent. Thus these two highly conserved isoleucines may play a defined role in stabilizing the interaction of the switch II region with the domain 1–domain 3 interface in the GDP complex. Interestingly, also in EF-G the homologous residues are conserved in the highly similar $\alpha 1$ part of the sequence. This makes it attractive to speculate that this structure, invisible in the EF-G•GDP crystal structure (Al-Karadaghi *et al.*, 1996), might also feature the helix-to-loop unrolling in the GDP to GTP transition.

The surprising increase in the intrinsic GTPase that was observed for mutation D80N in the isolated G-domain (Harmark *et al.*, 1992) is not found for the same substitution in the intact molecule, even in the presence of glycerol (not shown). Nevertheless, the reduction of the intrinsic catalysis is changed into a 5-fold stimulation on formation of a complex with aa-tRNA. This is in marked contrast with the wild-type situation, where normally binding aa-tRNA further reduces the GTPase, thus suppressing energy loss through unproductive GTP

hydrolysis. Moreover, empty ribosomes stimulate the mutant's GTPase to the same turnover rate as wt, as does kirromycin. This suggests that the overall efficiency of the (physiological) stimulated GTPase may not be negatively affected. However, the increased rate seen with aa-tRNA suggests that the mutation causes a conformational modification which makes the regulation of the GTPase less tight. In the absence of the constraints exercised by domains 2 and 3 on domain 1, this also leads to an increased intrinsic activity. The important influence of domains 2 and 3 on the conformation and regulation of domain 1 is also emphasised by the observation that the extreme lability that the G-domain[D80N] displayed is absent here.

Whereas the two substitutions of Thr-61 both impair the intrinsic GTPase, the complexation with aa-tRNA causes a small increase, though much less than for EF-Tu[D80N] and remaining below the wt intrinsic rate. The catalysis of both mutants is comparably enhanced by kirromycin, but ribosomes stimulate EF-Tu[T61A] much less than EF-Tu[T61N], which is activated to an extent approaching that of wt. This provides more evidence that GTPase activation by kirromycin and ribosomes follows different mechanisms (Krab & Parmeggiani, 1999a). The low ribosome stimulation of the EF-Tu[T61A] GTPase we find here was not observed for *T. thermophilus* EF-Tu_{Tt}[T62A] (Ahmadian *et al.*, 1995) at an assay temperature of 37 °C that is suboptimal for the thermophilic enzyme. The fact that even an Asn at this position—which may weakly bind the Mg²⁺ provided a conformational change to accommodate the longer side chain takes place—shows better activation by the ribosome indicates that for the ribosome-activated GTPase reaction the Thr-61 anchor point is important for the correct transition state configuration.

In poly(Phe) synthesis EF-Tu[D80N]'s function appears as much impaired as EF-Tu[T61A]. Nevertheless, in partial reactions such as enzymatic binding (at 10 mM Mg²⁺) the (empty) ribosome-stimulated GTPase it behaved similar to wt, whereas at least the ribosome-stimulated GTPase of EF-Tu[T61A] was slowed considerably. Thus, it seems that these two mutants are inhibited in different steps of the elongation cycle. For EF-Tu[D80N] the rate-limiting step may be the (re)formation of the ternary complex, due to the lower GTP affinity, whereas for EF-Tu[T61A] the slowest step may be the GTPase activation.

To resume, with the characterization of the mutants of Thr-25 published before and that of Thr-61 and Asp-80 presented in this work, we now have a fairly complete picture of the involvement of the network of magnesium binding residues in the GTPase. As a general trend we see that strong perturbation of the magnesium interaction reduces the intrinsic catalysis. This is in very good agreement with a transition state stabilization mechanism, in which the precise configuration of the various elements positioning the substrate and the nucleophile is essential. Whereas Thr-25 and Asp-80, both residues from “core” structural elements (helix α 1 and strand β 3, respectively), provide the main anchoring points for nucleotide binding, the peripheral effector region residue Thr-61 is weakly and transiently bound to the magnesium, playing however an important role in the transition state configuration. Inability of the residue at

position 61 to bind Mg^{2+} decreases but does not preclude activation by the ribosome, while on the other hand suppressing the link from position 25 did not seem to decrease stimulation. Asp-80 appears to be involved in the tight regulation of the GTPase. The proposed role of the hydrophobic barrier composed of residues Val-20 and Ile-60 is not supported by our data. This makes the case for His-84 being a catalytic residue activating the nucleophilic water even weaker. As for the possibility that His-84 orients the water molecule in the transition state of the ribosome-induced GTPase, an induced drastic conformation change in the effector region would still be a prerequisite, and more data on the way the ribosome is involved are necessary to understand the mechanism of the physiological ribosome-stimulated GTPase of EF-Tu.

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Chapter 5

Relevance of histidine-84 in elongation factor Tu GTPase activity and in poly(Phe) synthesis: its substitution by glutamine and alanine

Giuliana Scarano, Ivo M. Krab, Vincenzo Bocchini and Andrea Parmeggiani
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ABSTRACT

Substitution of His-84 ($\rightarrow$ Gln and $\rightarrow$ Ala), a residue of the switch II region of *E. coli* elongation factor (EF) Tu, hardly affected the binding of GTP or GDP. The activity in poly(Phe) synthesis and GTP hydrolysis of EF-Tu H84Q were both reduced to about 35%, as compared to EF-Tu wt, whereas EF-Tu H84A was inactive in poly(Phe) synthesis but still showed a 10% residual GTPase activity. Phe-tRNA^{Phe} exerted a similar inhibitory effect on the GTPase activity of EF-Tu wt and EF-Tu H84Q while abolishing that of EF-Tu H84A. Ribosomes enhanced the GTPase activity of EF-Tu H84Q, but not that of EF-Tu H84A, on which they even seemed to exert an inhibitory effect. The one-round GTP hydrolysis associated with the EF-TuH84Q-dependent binding of Phe-tRNA^{Phe} to poly(U)-programmed ribosomes was less efficient than with EF-Tu wt. Kirromycin stimulated the GTPase activities of both mutants less than EF-Tu wt. The results of this work do not support a catalytic role of His-84 in the intrinsic GTPase of EF-Tu, but they emphasize the importance of its side-chain for polypeptide synthesis and GTP hydrolysis.

1. Introduction

Histidine 84 is situated at the edge of the second consensus sequence element (*D80CPGH84*) of elongation factor (EF) Tu, that is part of the switch II region. This residue is conserved in elongation factors from different organisms, while in the family of ras proteins the corresponding position is occupied by glutamine (Dever *et al.*, 1987). The interest in His-84 rose from the finding that the EF-Tu-dependent GTP hydrolysis proceeds by an in-line attack of a molecule of water (Eccleston & Webb, 1982) and from the 3-D model of human H-ras p21, which suggested that the side-chain of Gln-61 (corresponding to His-84 in EF-Tu) could activate a water molecule found conveniently placed for a nucleophilic attack on the γ -phosphate of GTP (Pai *et al.*, 1990; Frech *et al.*, 1994). However, substitution H84G in the isolated GTP-binding domain of EF-Tu revealed a residual GTPase activity, making it unlikely that H84 is solely responsible for the catalysis (Cool & Parmeggiani, 1991).

In order to shed further light on its function, His-84 has been substituted by glutamine or alanine in the intact EF-Tu. The former substitution introduces the same residue as found in the homologous position in p21, which shares several properties with histidine including the ability of forming a hydrogen bond; the latter one investigates the effects due to the elimination of interactions but the hydrophobic ones. We did not introduce a glycine, as in the case of the G domain H84G, to avoid possible side-effects associated with the increased backbone flexibility of this residue.

The results show that the substitution H84Q is compatible with polypeptide synthesis and GTPase activity, whereas EF-Tu H84A can only sustain a low intrinsic GTPase activity. This emphasizes the importance of the nature of the side-chain in position 84 for a physiological function of EF-Tu in protein biosynthesis and the associated hydrolysis of GTP.

2. Materials and methods

The substitution of His-84 was performed by using 'unique-site elimination' mutagenesis (Deng & Nickoloff, 1992) on the *tufA* gene of EF-Tu *E. coli*, cloned into a pGEX2T vector (Smith & Johnson, 1988) in-frame with the gene encoding GST (Deng & Nickoloff, 1992). This method utilizes two primers, one to introduce the mutation, the other to eliminate one non-essential restriction site in the EF-Tu gene. The mutagenesis was essentially carried out by using the 'kit' and instructions of Pharmacia. The application of this method to the *tufA* gene will be described in detail elsewhere (I.M. Krab and A. Parmeggiani, in preparation). The overexpression of the GST-fused EF-Tu was induced with IPTG (0.3 mM) at a cell density of 0.6 Units_{600 nm}. Preparation of the cell extracts was carried out by sonication at 0 °C. EF-Tu fused with GST could be rapidly purified by affinity chromatography on glutathione-sepharose (Smith & Johnson, 1988), using 3.5 ml gel per cell extract from 1 liter culture. After washing with 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 30 mM NH₄Cl and 7 mM ME until no protein was released from the column, the cleavage of the fused GST-EF-Tu was obtained by passing

1 $\mu\text{g/ml}$ thrombin (Sigma) in the same buffer plus 2.5 mM CaCl_2 at a flow of 0.3-0.5 ml/min at 4 °C. The cleavage leaves EF-Tu with an extra glycine at the N-terminal end, that did not affect the action of EF-Tu (P. Anborgh, unpublished data). EF-Tu wt was also expressed as a GST-fusion and purified like the mutated factors. After thrombin-cleavage EF-Tu was chromatographed on a 5 ml Q-Sepharose column (Pharmacia). A wash with 50 ml of 180 mM KCl in 50 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 and 5 mM DTT containing 200 μM GDP to remove traces of EF-Ts, was followed by a linear KCl gradient (180-350 mM, 30 ml) in the same buffer containing 50 μM GDP and 1 mM DTT.

Poly(U)-directed poly(Phe) synthesis was followed kinetically by determining the amount of material insoluble in hot TCA (Swart *et al.*, 1987) and the GTPase activity by the liberation of $^{32}\text{P}_i$ using the charcoal method (Cr chet & Parmeggiani, 1986b). The NH_4Cl -washed 70 S ribosomes were prepared as described (Chinali *et al.*, 1977). Protein concentration was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. For further technical details see legends to figures.

3. Results

3.1. Purification and general properties of EF-Tu H84Q and H84A

The two mutated factors EF-Tu H84Q and H84A were expressed in *E. coli* almost as efficiently as EF-Tu wt; no anomalies in the cell growth rate were observed. The proteins were overproduced to about 20% of the total proteins of the cell extract. At least 50% of the mutated products remained soluble in the cell extract supernatant after a centrifugation for 15 min at $13,200 \times g$. The yield of the purified

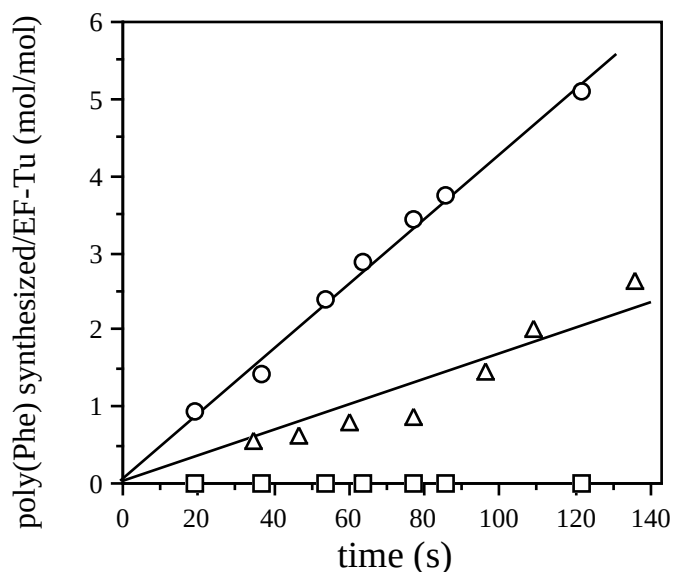


Fig. 1. Activities of EF-Tu wt (○), H84Q (Δ) and H84A (□) in poly(Phe) synthesis. Final reaction mixture (90 μl) contained: 50 mM Tris-HCl, pH 7.7, 7 mM MgCl_2 , 80 mM NH_4Cl , 5 mM ME, 30 pmol of EF-Tu wt or mutant, 1 mM PEP, 1.2 μg PK, 0.7 mM GTP, 1.5 mM ATP, 20 pmol EF-Ts, 50 pmol EF-G, 480 pmol $[^3\text{H}]\text{Phe}$ (specific activity 433 cpm/pmol), 450 pmol tRNA^{Phe} , Phe- tRNA^{Phe} synthetase, 50 pmol ribosomes with N-Ac-Phe- tRNA^{Phe} preloaded P-site and 7 μg poly(U). The reaction was started by adding EF-Tu•GTP. Samples (15 μl) were withdrawn at the indicated times and spotted on Whatman GF/A filters. The phenylalanine incorporation was measured by determining the radioactivity in the hot TCA-precipitate. EF-Tu•GTP was prepared by preincubation (15 min at 30 °C) with 3 mM PEP and 3.6 μg PK. Phe- tRNA^{Phe} was obtained by preincubating (30 °C for 30 min) 450 pmol tRNA^{Phe} (from a tRNA mixture containing 85% tRNA^{Phe}), 480 pmol $[^3\text{H}]\text{Phe}$ (specific activity as above) and saturating amounts of Phe- tRNA^{Phe} synthetase in 20 mM Tris-HCl, pH 7.8, 16 mM NH_4Cl , 8 mM MgCl_2 , 2 mM ATP and 2 mM DTT.

products was 8-10 mg per liter of culture. Their activities were stable for at least 1-2 months when kept at -20°C in 50 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 , 60 mM NH_4Cl , 10 μM GDP, 7 mM ME and 50% glycerol.

3.2. Effect of substitutions H84Q and H84A on poly(Phe) synthesis

As shown in Fig. 1, in a poly(Phe) synthesizing system, in which the ribosomal A-site was preloaded with N-Ac-Phe-tRNA^{Phe}, the activity of EF-Tu H84Q was reduced to about one-third that of EF-Tu wt. EF-Tu H84A was unable to sustain any phenylalanine incorporation.

3.3. Interaction with GDP and GTP

As observed in the case of the G domain H84G (Cool & Parmeggiani, 1991), the parameters governing the interaction between EF-Tu and GDP or GTP, especially the K_d -values, were not substantially changed in both EF-Tu H84Q and EF-Tu H84A, as compared to EF-Tu wt (Table 1).

Table 1

Effect of the substitution of His-84 by Gln or Ala on the GDP or GTP interaction of EF-Tu

System	K_d (nM)	$10^4 k_{-1}$ (s^{-1})	$k_{+1}(\text{s}^{-1}\cdot\text{mM}^{-1})$
EF-Tu wt•GDP	3.3	3.0	90
EF-Tu H84Q•GDP	3.8	6.8	180
EF-Tu H84A•GDP	2.8	8.0	290
EF-Tu wt•GTP	640	130	20
EF-Tu H84Q•GTP	580	200	50
EF-Tu H84A•GTP	510 ^a	220	40 ^a

The various parameters were measured at 0°C , as reported (Cool & Parmeggiani, 1991; Anborgh & Parmeggiani, 1993). The K_d was derived from the ratio k_{-1}/k_{+1} .

^a In the case of EF-Tu H84A, due to difficulties in measuring the k_{+1} , the K_d was determined directly by nitrocellulose binding assay using concentrations of [^3H]GTP (specific activity, 1437 dpm/pmol) from 0.115 to 7.0 μM ; the K_d -value was then calculated via a Scatchard plot (Cr  chet & Parmeggiani, 1986a).

3.4. GTPase activity

EF-Tu is a GTPase with a very low intrinsic activity. The antibiotic kirromycin enhances this activity (Parmeggiani & Swart, 1985). As shown in Fig. 2A, the GTPase activities of the two mutated EF-Tu's were decreased in comparison to that of EF-Tu wt. The activity of EF-Tu H84Q was around 35% that of EF-Tu wt, whereas the GTPase of EF-Tu H84A was only 10%. Kirromycin had more influence on the GTPase of EF-Tu wt than on that of EF-Tu H84Q and EF-Tu H84A. Its effect was, in the chosen conditions, to stimulate the GTPase activity of EF-Tu wt by almost 8 times, and that of EF-TuH84Q and EF-TuH84A by about 3 times (Fig. 2B).

Phe-tRNA^{Phe} reduced the GTPase activity of EF-Tu wt and of EF-TuH84Q by ~50%, while no activity could be detected with EF-Tu H84A (Fig. 3A). The inhibition of the intrinsic GTPase activity of EF-Tu upon binding of aa-tRNA is a well-known effect (Fasano *et al.*, 1982b; Picone & Parmeggiani, 1983) and likely to be physiologically important for conserving the integrity of the carrier of aa-tRNA to the ribosome. Kirromycin reversed the inhibitory effect of aa-tRNA on the intrinsic GTPase of EF-Tu into a marked stimulation; 30–40 times in the case of EF-Tu wt vs. only 10 times in the case of EF-Tu H84Q (Fig. 3B), whereas no activity at all was observed with EF-Tu H84A. In an assay measuring the kinetics of the

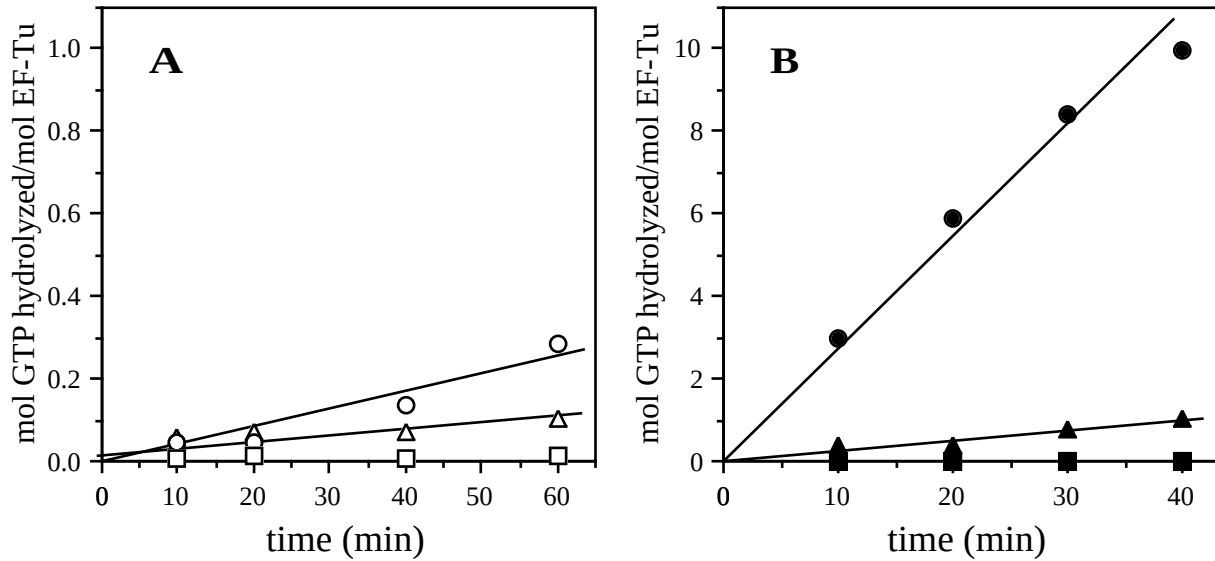


Fig. 2. The intrinsic GTPase activity of EF-Tu wt (○,●), H84Q (△,▲) or H84A (□,■) in the absence (A, open symbols) and the presence of kirromycin (B, filled symbols). (A) Final reaction mixture (90 μ l): 50 mM Tris-HCl, pH 7.7, 10 mM MgCl_2 , 100 mM NH_4Cl , 1 mM DTT, 108 pmol EF-Tu wt or mutant, 1350 pmol $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (specific activity 1700 cpm/pmol). The reaction was started by the addition of EF-Tu $\cdot$ $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (prepared as described in the legend to Fig. 1) at 30 $^\circ\text{C}$. Samples (20 μ l) were withdrawn at the given times and the $^{32}\text{P}_i$ liberated was measured. (B) With kirromycin (50 μM) the amount of EF-Tu was 72 pmol and of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (specific activity 1280 cpm/pmol) 9180 pmol.

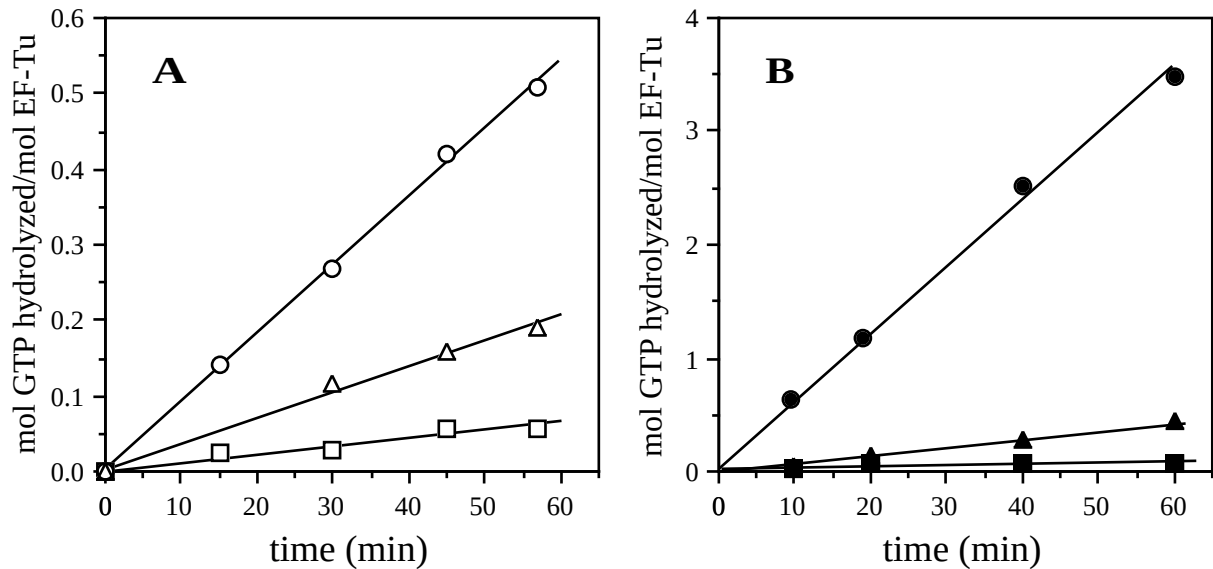


Fig. 3. Effect of Phe-tRNA^{Phe} on the GTPase activity of EF-Tu wt (○,●), H84Q (△,▲) or H84A (□,■) without (A, open symbols) and with kirromycin (B, filled symbols). (A) Final reaction mixture (90 μ l): 72 pmol of either wild-type or mutated EF-Tu, 144 pmol Phe-tRNA^{Phe}, 1400 pmol $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (specific activity 1252 cpm/pmol) in the same buffer as in Fig. 2. The reaction was started by the addition of EF-Tu $\cdot$ $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (see legend to Fig. 1) at 30 $^\circ\text{C}$. tRNA^{Phe} was aminoacylated as reported in the legend to Fig. 1, except that 500 pmol tRNA^{Phe} (from a tRNA mixture containing 1.8% tRNA^{Phe}) and 1000 pmol Phe were used. At the given times, samples (20 μ l) were withdrawn and the $^{32}\text{P}_i$ liberated measured. (B) With kirromycin (50 μM), the amount of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was 1700 pmol (specific activity 1500 cpm/pmol).

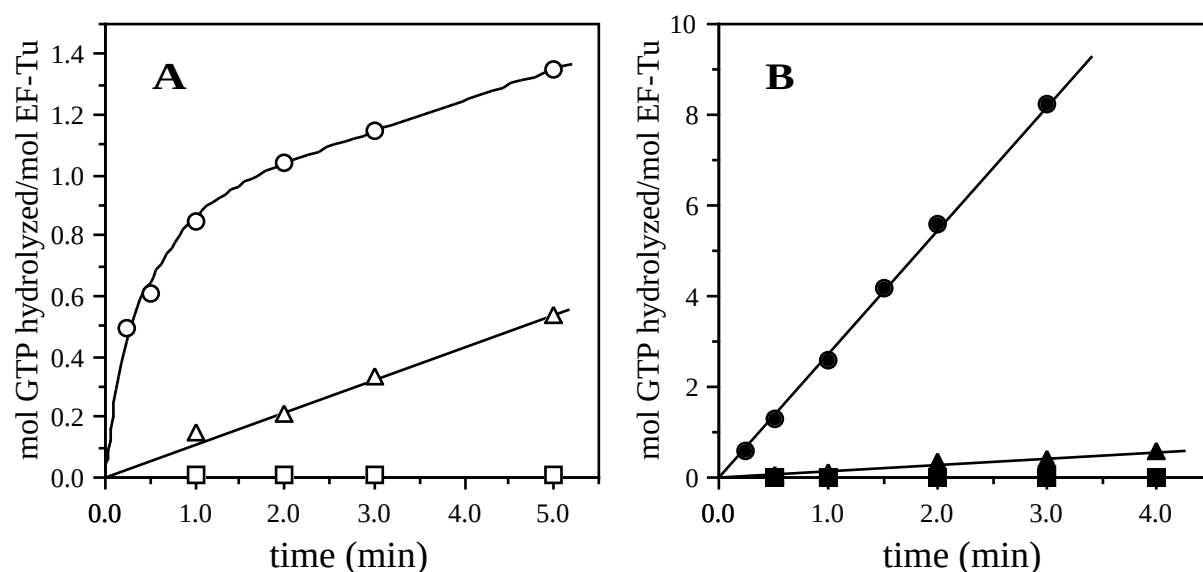


Fig. 4. The GTPase activity of EF-Tu wt (○,●), H84Q (△,▲) or H84A (□,■) in the presence of ribosomes without (A, open symbols) and with kirromycin (B, filled symbols). (A) Final reaction mixture (120 μl): 96 pmol of EF-Tu wt or mutant, 192 pmol ribosomes, 1200 pmol of [γ - ^{32} P]GTP (specific activity 560 cpm/pmol) in 50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 100 mM KCl and 7 mM ME. The reaction was started at 30 °C by the addition of ribosomes. The liberated ^{32} P_i was determined on 20 μl samples. EF-Tu•[γ - ^{32} P]GTP was prepared as reported in the legend to Fig. 1. (B) With kirromycin (50 μM), the amount of [γ - ^{32} P]GTP used for the EF-Tu reaction mixture was 3000 pmol with a specific activity of 460 cpm/pmol.

spontaneous deacylation of Phe-tRNA^{Phe} under similar experimental conditions, EF-Tu H84A stabilized the ester bond of the aa-tRNA with efficiency indistinguishable from that of EF-Tu wt, whereas EF-Tu H84Q was slightly less efficient (data not shown).

The results obtained with the addition of non-programmed ribosomes to the preformed EF-Tu•[γ - ^{32} P]GTP complex are illustrated in Fig. 4A. The GTPase of EF-Tu wt showed an initial fast phase, corresponding to 0.8-0.9 mol GTP hydrolyzed per mol EF-Tu (Fasano *et al.*, 1982b; Picone & Parmeggiani, 1983). The ribosomes also enhanced the GTPase activity of EF-Tu H84Q but in this case the initial burst was either not present or much reduced (the latter effect not illustrated). In contrast, the turnover GTPase of EF-Tu H84Q displayed the same rate as EF-Tu wt. These results show that the stimulation of the catalytic activity of EF-Tu H84Q•GTP by the ribosome is less efficient than with EF-Tu wt, as shown by the deficient initial phase. Unlike this, the rate of the turnover GTPase of EF-Tu wt and EF-Tu H84Q is similar, since the rate-limiting step is now the regeneration of the EF-Tu•GTP complex rather than the GTP hydrolysis (Fasano *et al.*, 1982b; Picone & Parmeggiani, 1983) and the interaction with GTP and GDP is hardly changed by mutation H84Q (Table 1). Kirromycin eliminated the difference between initial and turnover GTPase activity of EF-Tu wt•GTP (Fig. 4B), the activity corresponding to the initial velocity in the absence of the antibiotic (Fig. 4A and B). This effect is a consequence of the property of kirromycin to change the rate-limiting

step in the EF-Tu GTPase, which is no longer the regeneration of the EF-Tu•GTP complex but the breakdown of GTP, due to the strong stimulation of the GDP/GTP exchange by the antibiotic (Bocchini *et al.*, 1980). Ribosomes did not enhance the GTPase activity of EF-Tu H84A, on which they even appeared to exert an inhibitory effect. Kirromycin did not change this picture.

With poly(U)-programmed ribosomes plus Phe-tRNA^{Phe}, i.e. under conditions leading to the EF-Tu•GTP-mediated binding of Phe-tRNA^{Phe} to the ribosomal A-site, EF-Tu wt displayed a burst-like hydrolysis of GTP, corresponding to a 1-to-1 stoichiometric ratio with respect to the molar amount of EF-Tu (Fig. 5). Also EF-Tu H84Q showed an initial burst-like activity that was, however, only one-third that of EF-Tu wt, after which the extent of GTP hydrolyzed steadily increased to a level close to that of EF-Tu wt. In experiments not illustrated, the kinetics of binding of Phe-tRNA^{Phe} to the poly(U)-ribosome mediated by EF-Tu H84Q gave the same results as EF-Tu wt.

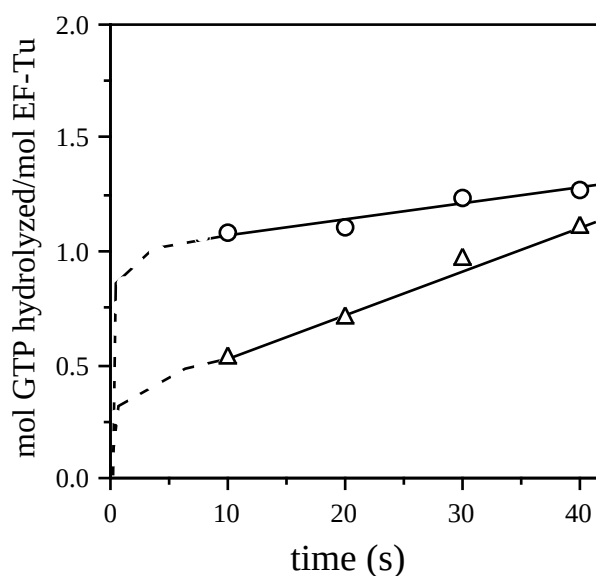


Fig. 5. The GTPase activity of EF-Tu wt (○) and H84Q (△) in the presence of Phe-tRNA^{Phe} and poly(U)-programmed ribosomes. Final reaction mixture (65 µl): 50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 100 mM KCl, 72 pmol EF-Tu, 346 pmol ribosomes, 346 pmol Phe-tRNA^{Phe}, 168 µg poly(U), 918 pmol [γ -³²P]GTP (specific activity 1812 cpm/pmol) and 1 mM DTT. EF-Tu•[γ -³²P]GTP and Phe-tRNA^{Phe} (from a tRNA mixture containing 1.8% tRNA^{Phe}) were prepared as described in the legend to Fig. 1. The reaction was started at 30 °C by the addition of poly(U)-programmed ribosomes preloaded with tRNA^{Phe} in the P-site. Samples (20 µl) were withdrawn at the given times and the liberated ³²P_i was measured. When this assay was performed with 85% pure tRNA^{Phe} or with ribosomes preloaded with N-AcPhe-tRNA^{Phe}, similar results were obtained.

4. Discussion

The high resolution 3-D model of the complex of EF-Tu *Thermus Thermophilus* with GMPPNP shows that in EF-Tu there is a water molecule that could act as nucleophile on the γ -phosphate of GTP (Berchtold *et al.*, 1993). However, the existence of a 'hydrophobic gate', formed by Val-21 and Ile-61 (Val-20 and Ile-60, respectively, in *E. coli* EF-Tu), situated between His-85 (His-84 in *E. coli*) and this water molecule makes an activation of the water molecule by the side-chain of His-85 improbable at least for the slow intrinsic GTPase activity of EF-Tu. Differently from EF-Tu, in one of the conformations of the flexible switch II region of p21, the side-chain of Gln-61 points to a water molecule in line with the γ -phosphate of GTP, without the interference of a hydrophobic barrier (Pai *et al.*, 1990). In p21, the possibility that Gln-61 activates the nucleophilic water molecule was questioned (Langen *et al.*, 1992; Privé *et al.*, 1992). Langen *et al.* (Langen *et al.*, 1992) suggested, on the basis of theoretical considerations, that the γ -phosphate itself could act as a proton acceptor and that the role of Gln-61 would be only to stabilize the nucleophile by a hydrogen bond. Very recently experimental evidence has been described supporting this model (Schweins *et al.*, 1995), that could also be in part valid for the intrinsic GTPase of EF-Tu (Hilgenfeld, 1995). However, a different situation is likely to underlie the ribosomal EF-Tu-dependent GTP hydrolysis. The interaction of EF-Tu•GTP with mRNA-programmed ribosomes during the binding of aa-tRNA can increase the velocity of the GTPase reaction by 5 orders of magnitude, as derived from experiments reported in (Fasano *et al.*, 1982b; Bilgin *et al.*, 1988). In these conditions, the need for an activation mechanism is evident.

A number of conclusions can be drawn from the results of this work. Whereas the binding of GTP or GDP is little or not affected by substitutions H84A and H84Q, the GTPase activity of EF-Tu is modified under all conditions tested. This emphasizes the relevant role of H84 in this activity. The presence of a protonated side-chain of the 84 residue, though important, does not appear to be essential for the intrinsic GTPase of EF-Tu, as shown by the low but significant hydrolysis sustained by EF-Tu H84A. In this regard, the action of alanine resembles that of glycine in the isolated EF-Tu GTP-binding domain carrying the substitution H84G (Cool & Parmeggiani, 1991). The residual GTPase activity observed with EF-Tu H84A is compatible with the recent model for the p21 intrinsic GTPase (see above and: Langen *et al.*, 1992). However, for an efficient function of EF-Tu in the process of elongation, the properties of the side-chain of histidine become essential. Glutamine shares only in part the properties of histidine, as shown by the lower activity of EF-Tu H84Q in poly(Phe) synthesis and GTPase, whereas alanine is unable to substitute for histidine in the physiological functions. Whether the specific properties of the side-chain of histidine concern its ability to abstract a proton, the resulting positive charge or its capability of forming a hydrogen bond, remains to be clarified.

Concerning the macromolecular ligands of EF-Tu, both the experiments in Figs. 4 and 5 indicate that the ribosome does not activate the EF-Tu H84A GTPase but can induce a

catalytically active state of EF-Tu H84Q, though less efficient than that of EF-Tu wt. In the presence of μM concentrations of Phe-tRNA^{Phe}, that are largely saturating (the affinity of aa-tRNA for EF-Tu•GTP lies in the nmolar range (Johnson *et al.*, 1985)), the GTPase of both mutants is lowered, in EF-Tu H84A practically abolished. In the case of EF-Tu H84Q the slower GTP hydrolysis accompanying this reaction is not due to a defective interaction with aa-tRNA, but rather to a negative influence on the catalytically active state, since this mutant can still effectively support the enzymatic binding of Phe-tRNA^{Phe} as well as protect the ester bond of Phe-tRNA^{Phe} against spontaneous deacylation.

To conclude, in elongation the action of the mRNA-programmed ribosome on EF-Tu•GTP•aa-tRNA may induce such a dramatic conformational change of the factor as to allow the efficient activation of a water molecule as a nucleophile by an as yet unidentified residue that could be represented by His-84. Alternatively, His-84 could perform an important indirect action in GTP hydrolysis, as suggested for the intrinsic GTPase (Cool & Parmeggiani, 1991), by favoring the active state of the GTP binding pocket of EF-Tu.

Acknowledgements

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Chapter 6

Elongation factor Ts can act as a steric chaperon by increasing the solubility of nucleotide binding-impaired EF-Tu

Ivo M.Krab, Rob te Biesebeke, Alberto Bernardi and Andrea Parmeggiani

(submitted to Biochemistry)

ABSTRACT

Several EF-Tu mutants that have impaired nucleotide binding show decreased solubility on overexpression in the *E. coli* cell, an indication that they do not fold correctly. Moreover, EF-Tu[T25A] and EF-Tu[D80N] were shown to inhibit cell growth on expression, an effect attributed to their sequestration of EF-Ts. We present here results showing that the co-overexpression of EF-Ts at a 1:1 ratio dramatically improves the solubility of mutant EF-Tu, although in the case of EF-Tu[D138N] —which cannot at all bind the nucleotides available in the cell— this is a slow process. Moreover, the mentioned growth inhibition is relieved. We conclude that for the formation of a correct EF-Tu structure the nucleotide plays an important role as a “folding nucleus”, and that also in its absence EF-Ts can act as a folding template or steric chaperon for the correct folding of EF-Tu.

INTRODUCTION

Bacterial elongation factor (EF) Tu is a GTPase that transports aminoacyl (aa-)tRNAs to the mRNA-programmed ribosome in the elongation cycle of protein synthesis (reviewed in: Krab & Parmeggiani, 1998), accelerating dramatically the elongation process and contributing to its high selectivity. It binds guanosine di- and triphosphate—but not the monophosphate (Miller & Weissbach, 1977)—with high affinity and possesses an intrinsic ability to catalyse γ -phosphate hydrolysis of bound GTP. This catalytic activity is increased by approx. 5 orders of magnitude when the transported aa-tRNA has codon-anticodon interaction with the mRNA in the A site of the elongating ribosome. EF-Tu from *Escherichia coli* is only moderately temperature stable *in vitro* (rapidly inactivated at temperatures above 45 °C) when bound to a GDP or GTP nucleotide (Anborgh *et al.*, 1992; Anborgh & Parmeggiani, 1993) and unstable in the absence of bound nucleotide (Fasano *et al.*, 1982a). On the other hand, the complex with its guanine nucleotide exchange factor EF-Ts is stable in the absence of nucleotide for extended periods of time (Parmeggiani & Sander, 1981). EF-Tu with the substitution Asp138→Asn specifically binds xanthosine nucleotides (di- or triphosphate) instead of guanine nucleotides (Hwang & Miller, 1987). It was used as a tool to investigate the energy consumption by EF-Tu during elongation (Weijland & Parmeggiani, 1993; Weijland *et al.*, 1994). Because it hydrolyzes XTP specifically, the NTP consumption by EF-Tu becomes distinguishable from that of other protein synthesis factors. Unfortunately, the yield of soluble protein after overexpression of this mutant protein in *E. coli* is very low, making it laborious and time-consuming to isolate it in significant amounts. Noteworthy, although xanthosine and its monophosphate form are present in the cell as intermediates in the pathway of guanosine nucleotide synthesis, XDP and XTP are not. Although any NDP will be converted to NTP by nucleoside diphosphokinase, the formation of nucleoside diphosphates from the monophosphates is catalysed by base-specific enzymes (adenylate kinase, cytidylate kinase, guanylate kinase, uridylate kinase and deoxythymidylate kinase) (Neuhard & Nygaard, 1987). However, no xanthosylate kinase exists in *E. coli* (Neuhard & Nygaard, 1987) and the consequent lack of polyphosphorylated xanthosyl nucleotides in the cell makes EF-Ts the only possible source of stabilization of EF-Tu[D138N]. Indeed, the maximum level of this mutant found in the soluble fraction on overexpression never exceeds 1/10th that of wt EF-Tu in the cell, which corresponds closely to the level of cellular EF-Ts (Albert Weijland, private communication).

This observation prompted us to investigate if the solubility of EF-Tu[D138N] was somehow limited by the amount of EF-Ts available. For this purpose, we constructed a vector which allows simultaneous overexpression of glutathione-S-transferase (GST)-fused EF-Tu (Smith & Johnson, 1988) and of EF-Ts. This vector was then used to not only express GST-EF-Tu[D138N], but also other EF-Tu mutants with strongly decreased affinity for guanine nucleotides, which limits their solubility on overexpression similarly to that of EF-Tu[D138N].

MATERIALS AND METHODS

Vector pTuTs was derived from pGtAM-wt, a pGEX-2T (Smith & Johnson, 1988) derivative expressing GST-fused *E. coli* EF-TuA (Krab & Parmeggiani, 1999b). First the entire *tufA* gene was PCR amplified using an oligonucleotide that hybridizes within the GST coding sequence and one that hybridizes to the end of the *tufA* coding sequence, introducing a Pst-site adjacent to the stop codon. This fragment was cloned BamHI-PstI into a pGEX-2T-derivative that has a Pst site instead of the EcoRI site (A. Bernardi, unpublished). Then the BamHI-NcoI fragment comprising the major part of the EF-Tu sequence was replaced by that from pGtAM-wt, to avoid amplification errors in this region. The remaining part of the EF-Tu open reading frame was verified by sequencing. Next, a 1.26 kB SmaI-PstI fragment from pTS21 (a gift of Dr. Yu Wen-Hwang) containing the *E. coli tsf* gene was inserted using a PstI linker on the SmaI blunt end. A 200 bp spacer devoid of transcription stops separates the EF-Tu and EF-Ts open reading frames. pTu[D138N]Ts was derived from this vector by transferring the 240 bp AccI-AccI *tuf* gene-internal fragment from pGtA-D138N into pTuTs. pTu[T25A]Ts, pTu[H22Y/T25S]Ts and pTu[D80N]Ts were made by transferring the 760 bp BamHI-partial AccI fragment from the respective GST-EF-Tu expressing vectors (see Table I).

For overexpression studies cells were grown in LB medium with 100 µg/ml ampicilline at the indicated temperatures and induced with either 300 or 75 µM isopropylthiogalactoside. The development of the optical density at 600 nm was followed in time. At specified times cells from samples of 20 ml culture per OD_{600 nm} were collected by centrifugation and resuspended in 1.3 ml of lysis buffer (50 mM Tris•HCl pH 7.6, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 10% glycerol and 0.5 mg/ml lysozyme), incubated on ice for 30-45 min and then sonicated for 2 min total time. A sample of the soluble fraction was separated from insoluble debris by centrifugation at 13000 rpm in a microfuge for 10 min. The pellet was resuspended in an equal volume of cracking buffer. 15 µl of supernatant and an equivalent amount of resuspended pellet were analyzed on SDS-PAGE.

RESULTS

High-level simultaneous overexpression of GST-EF-Tu and EF-Ts from pTuTs

In order to obtain simultaneous overexpression of GST-EF-Tu and EF-Ts in stoichiometric amounts, we constructed vector pTuTs (see Materials and Methods), which unites both genes in the same operon under the control of the p_{tac} promoter. The induction of expression by isopropylthiogalactoside resulted in very high overexpression of both GST-EF-Tu and EF-Ts in the cell at approximately equal levels, in soluble amounts even somewhat better than the overexpression of EF-Tu alone (not illustrated). We subsequently made derivatives containing different EF-Tu mutations to see the effect of the coexpression of EF-Ts on their solubility (see Table I).

Table I — Plasmids used in this study		
Plasmid name	Expressed proteins	Reference
pGtAM-wt	GST-EF-Tu wt	(Krab & Parmeggiani, 1999b)
pTuTs	GST-EF-Tu wt + EF-Ts	
pGtAp-T25A	GST-EF-Tu[T25A]	(Krab & Parmeggiani, 1999a)
pTu[T25A]Ts	GST-EF-Tu[T25A] + EF-Ts	
pGtAp-H22Y/T25S	GST-EF-Tu[H22Y/T25S]	(Krab & Parmeggiani, 1999a)
pTu[H22Y/T25S]Ts	GST-EF-Tu[H22Y/T25S] + EF-Ts	
pGtA-D80N	GST-EF-Tu[D80N]	(Krab & Parmeggiani, 1999b)
pTu[D80N]Ts	GST-EF-Tu[D80N] + EF-Ts	
pGtA-D138N	GST-EF-Tu[D138N]	(Weijland <i>et al.</i> , 1994)
pTu[D138N]Ts	GST-EF-Tu[D138N] + EF-Ts	

Overexpression and solubility of xanthosine nucleotide-specific EF-Tu[D138N]

When we induced expression of pTu[D138N]Ts we expected to see an increase in the fraction of EF-Tu[D138N] that is present in the supernatant of an S30 extract. However, at short incubation times (<6 h) we did not observe any significant increase in solubility compared to the overexpression of EF-Tu[D138N] alone. On the other hand, the overexpressed EF-Ts in that case was very soluble. Nevertheless, we were encouraged by the finding that the supernatant contained small but increasing amounts of soluble GST-EF-Tu[D138N] at longer induction periods (up to 22 hrs). In contrast, in the absence of coexpression of EF-Ts no significant solubilization was found at any time. Combining the coexpression of EF-Ts with a decrease in the induction temperature (from 30 °C to 24 °C), which has in general a beneficial effect on solubility of overexpressed proteins, we obtained the result shown in **Fig. 1A**: After 7 h of induction with 0.3 mM IPTG already high amounts of both EF-Tu and EF-Ts have been produced, but only part of the GST-EF-Tu[D138N] is found in the supernatant unlike the EF-

Ts, which is mostly soluble. However, after 32 h, well into the stationary growth phase, approximately 50% of the GST-EF-Tu is soluble. After 70 h when the cells are hardly growing anymore very high amounts of soluble mutant protein are obtained: approximately 20 mg of pure thrombine-cleaved EF-Tu/EF-Ts complex per liter of culture. These results emphasize the difficulty that the EF-Tu molecule has to achieve its native fold in the absence of a suitable nucleotide to serve as a “folding nucleus” and show that EF-Ts can, probably through a template action, assist in the folding process. To verify if the observed increase in soluble EF-Tu—in **Fig. 1A** not observably accompanied by a decrease in the amount of insoluble factor—is just due to the high concentration of EF-Ts in the cytoplasm assisting in the folding of newly transcribed EF-Tu, or if insoluble factor can still refold and go into solution in the presence of EF-Ts, we did the control experiment in **Fig. 1B**. After 8 h of induction a sample was analyzed for solubility and at the same time further protein synthesis was stopped by the addition of 170 µg/ml of chloramphenicol to the growth medium. The sample analyzed after 25 h shows that almost all of the EF-Tu that at first was insoluble, now is present in the supernatant, showing that, indeed, refolding of precipitated EF-Tu takes place

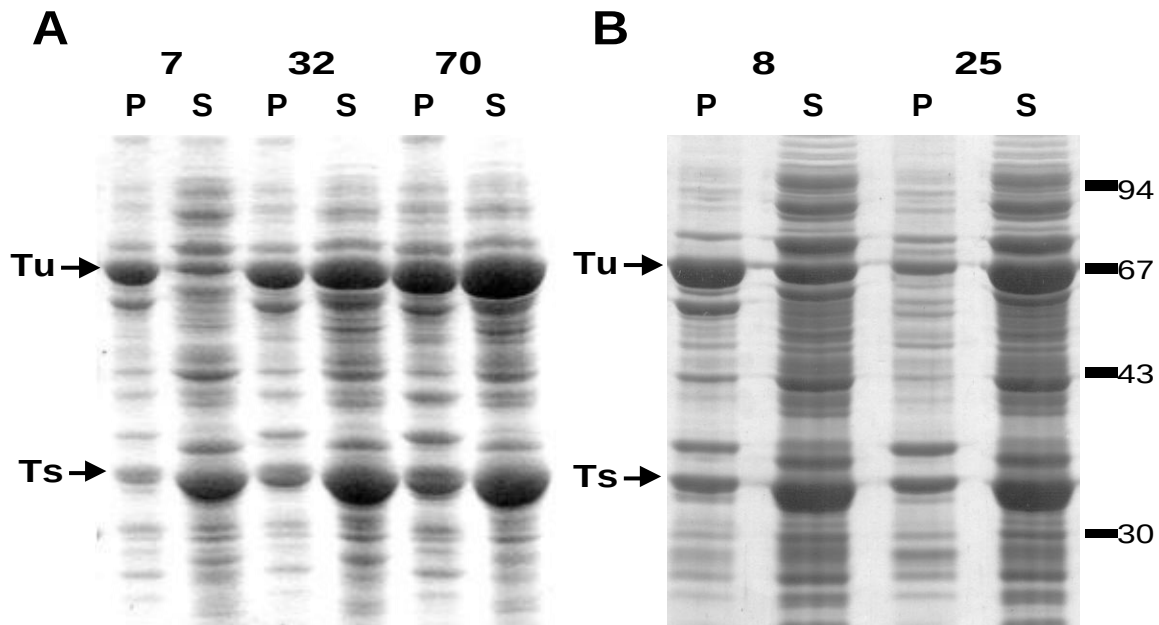


Figure 1. Long induction of pTu[D138N]Ts. Panel **A**: SDS-PAGE showing S30 supernatant (S) and pellet (P) fractions at the indicated time after induction of expression by isopropylthiogalactoside at 24 °C. The position of GST-EF-Tu[D138N] is indicated by an arrow marked **Tu** and that of EF-Ts by **Ts**. Panel **B**: idem for cells harvested after 8 h induction during normal growth at 25 °C, after which protein synthesis was arrested using chloramphenicol and another sample was analyzed after 25 h.

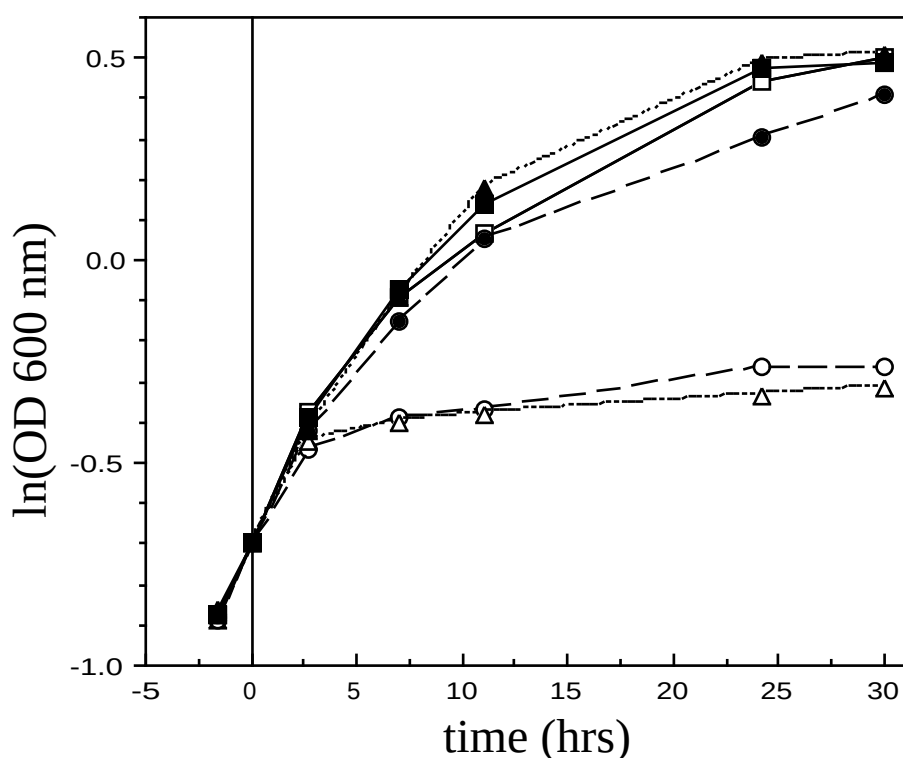


Figure 2. Increase of cell density during induction of mutant EF-Tu with and without coexpression of EF-Ts. Plot of $^{10}\log(\text{OD}_{600 \text{ nm}})$ of LB cultures at different times relative to the time of induction (at an $\text{OD}_{600 \text{ nm}}$ value of 0.2) of the plasmids expressing GST-EF-Tu wt ($\square, \blacksquare$), GST-EF-Tu[T25A] ($\Delta, \blacktriangle$) and GST-EF-Tu[D80N] ($\circ, \bullet$) without (open symbols) or with (filled symbols) coexpression of EF-Ts.

Growth inhibition by EF-Tu[T25A] and EF-Tu[D80N] is relieved by overexpression of EF-Ts

We have recently shown that EF-Tu carrying the substitutions T25A or D80N display an increased stability of the complex with EF-Ts which cause them to interfere *in vitro* with the dissociation of wt EF-Tu•GDP by EF-Ts (Krab & Parmeggiani, 1999b; Krab & Parmeggiani, 1999a). The new vector coexpressing EF-Ts allowed to compare the *in vivo* effect of these mutants in the presence of normal (limiting) amounts of EF-Ts and in the presence of equal amounts of the exchange factor. As illustrated in **Figure 2**, overexpression of GST-EF-Tu[T25A] or GST-EF-Tu[D80N] alone strongly decreases the growth rate, whereas 1:1 coexpression of EF-Ts completely relieves this inhibition. Thus these mutated EF-Tu have a dominant negative behaviour towards the wild type EF-Tu in the cell in the presence of limiting amounts of EF-Ts, an effect that can be attributed to sequestering of the available EF-Ts by the mutants. In fact, since GST-fused EF-Tu can only to a small extent (~5 %) participate in protein synthesis (Anborgh, 1993), it is unlikely that the growth-inhibiting effect is due to direct interference of the mutant GST-EF-Tu with the protein biosynthetic process. In line with this, the other mutants that did not show *in vitro* interference with EF-Ts stimulation of wt EF-

Tu•GDP dissociation, also did not inhibit growth (not shown). Even expression of GST-EF-Tu[D138N]—whose complex with EF-Ts, once formed, cannot be dissociated in the cell for lack of XDP/XTP— did not retard growth. This is in agreement with the observed slow solubilization of EF-Tu[D138N] even in the presence of excess EF-Ts and implies that the correct folding of the factor is so slow that sequestering of EF-Ts does not occur while the cells are still actively growing.

Overexpression and solubility EF-Tu mutants with low affinity for guanine nucleotides

The two mutants mentioned above, and a third one, the double mutant H22Y/T25S also displayed a low yield of soluble protein on overexpression. All these mutant EF-Tu display a strongly decreased affinity for guanine nucleotides. For EF-Tu[T25A] and EF-Tu[D80N] the low yield is in part attributable to their dominant negative effect described above, causing inhibition of further protein synthesis. This is in part also due to lower solubility of the factors. **Figure 3** illustrates this for mutants T25A and double mutant H22T/T25S. At short times after the start of induction (<5 hrs), only a fraction of the GST-EF-Tu[T25A] is found in the supernatant (**Figure 3A**). At longer times the solubility increases to more than 50%, but the

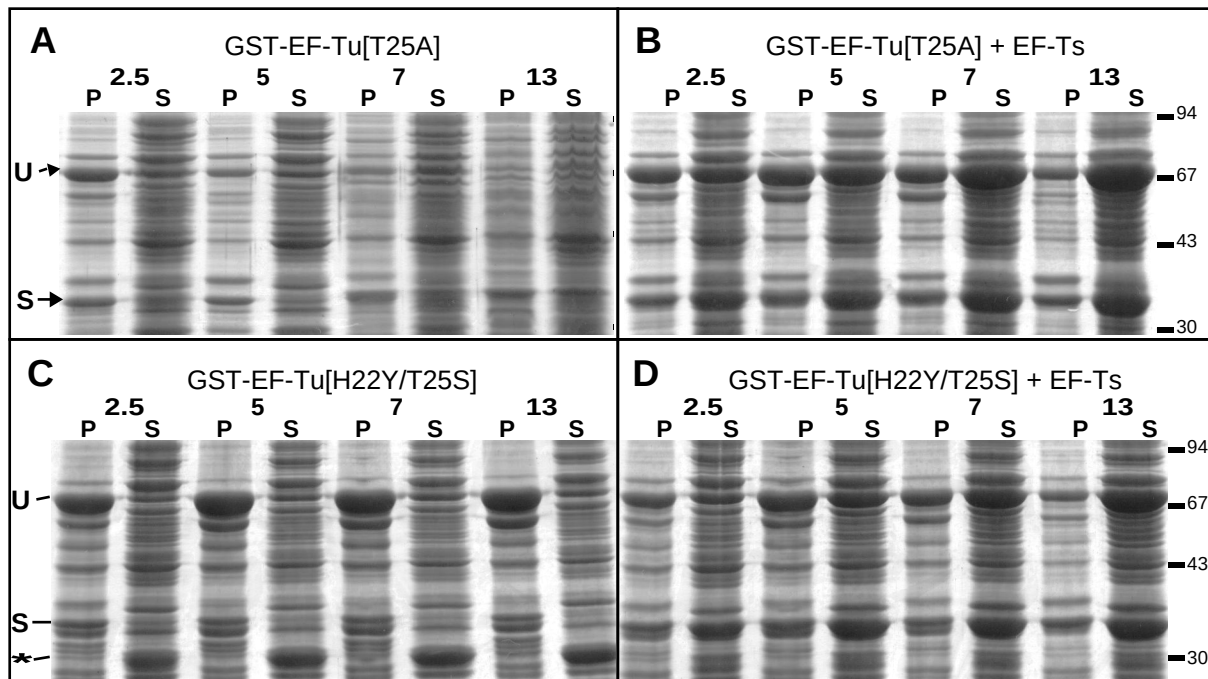


Figure 3. Improved production and solubility of mutant EF-Tu when co-overexpressing EF-Ts. SDS-PAGE showing S30 supernatant (S) and pellet (P) fractions at the indicated time after induction of expression by isopropylthiogalactoside at 24 °C. The position of GST-EF-Tu is indicated by an arrow marked U and that of EF-Ts by S. The arrow marked with an asterisk indicates the position of the approximately 30 kD soluble degradation product discussed in the text. The induction of GST-EF-Tu[T25A] gave rise to lysis phenomena at longer times coinciding with fuzzyness of the protein bands on gel.

total amount of protein does not further increase. The protein extracts show a characteristic fuzziness of the bands on SDS-PAGE at longer induction times, which coincides with the occurrence of spontaneous lysis in the cultures. The double EF-Tu mutant H22Y/T25S is expressed at a much higher level, but the amount of full-length product in the supernatant remains low (**Figure 3C**). Moreover it suffers from a degradation problem: an approximately 30 kD band increases in intensity in the supernatant **Figure 3C**. At longer expression times (>20 h) this band increases strongly in intensity, while at the same time the band of full-length product disappears from the pellet (not shown). For both mutants coexpression of EF-Ts dramatically improves the fraction of GST-EF-Tu that is present in the supernatant, although it takes several hours before the majority of the product is found in the soluble fraction (**Figure 3B and D**). Note that the ~30 kD band due to degradation is not detectable in **Figure 3D**. Thus, EF-Ts is able to assist folding of EF-Tu also for mutants which do not bind nucleotides strongly enough to induce and maintain their native structure in the cell.

DISCUSSION

We have observed recently that several mutants incapable of or defective in binding the nucleotides available in the cell are only partially soluble on overexpression. EF-Tu from *E. coli* is not a very stable protein. Temperature-induced inactivation occurs at relatively low temperatures: $\theta_{1/2}$, the temperature at which half of the EF-Tu is inactivated after 8 min is ~52 °C for EF-Tu•GDP and ~47 °C for EF-Tu•GTP (Anborgh *et al.*, 1992). Nucleotide-free EF-Tu from *E. coli* is even less stable *in vitro* and rapidly loses activity at 4 °C. This loss of activity, however, can in certain conditions be reversed up to a certain point by addition of GDP; this reactivation is accelerated by the antibiotic kirromycin (Fasano *et al.*, 1982a). On the other hand the nucleotide-free complex with EF-Ts is very stable on the long term (Parmeggiani & Sander, 1981). Clearly, the interactions of EF-Tu with the nucleotide and the bound magnesium, or in their absence with EF-Ts, are essential for maintaining a correctly folded state of EF-Tu. We suspected, therefore, that the low solubility of these mutants and their reduced nucleotide binding are connected. Correct folding of EF-Tu in the cellular environment could be dependent on a nucleotide acting as a template for the (re)folding of the protein or at least stabilizing a spontaneously correctly folded protein. In absence of a suitable nucleotide the only other possible stabilizing factor would be EF-Ts, which is present in a lower concentration than EF-Tu in normal circumstances (approximately 1/10 of the EF-Tu concentration: Gouy & Grantham, 1980).

In this study we have investigated the effect of simultaneous overexpression of GST-fused EF-Tu and EF-Ts on the yield of soluble GST-EF-Tu in the postribosomal supernatant fraction. We observed a general beneficial effect on the solubility of the expressed proteins. Even the

overexpression level of soluble wt EF-Tu seems somewhat improved (not shown), but the effect is more obvious for mutants T25A, H22Y/T25S and D80N which have a reduced nucleotide affinity. Most striking is the effect for mutant D138N, which cannot bind any nucleotide available in the cell and is very little soluble in normal circumstances. Differently from the other mutants, however, significant formation of soluble product occurs only well into the stationary phase, while EF-Ts is immediately mostly soluble. In fact, even initially insoluble EF-Tu can go into solution on continued incubation when new protein synthesis has been arrested with chloramphenicol. This is indicative of a slow conformational rearrangement of initially misfolded and insoluble protein, which can form a stable, soluble complex with EF-Ts once the (near)-native fold is achieved. Thus, EF-Ts appears to act as a folding template, a steric molecular chaperon (as defined by: Ellis, 1998) for the folding of EF-Tu. For the other mutants, the residual nucleotide-binding ability seems to allow the nucleotide to act as a folding template up to a certain point, but the lower affinity limits its effectiveness and/or decreases the *in vivo* stability of the resulting protein. Consequently a 1:1 stoichiometry of EF-Ts is necessary for maximum solubility and long term stability.

As a second important finding we here show that mutants T25A and D80N display *in vivo* a dominant negative phenotype, similar to that of its Ras p21 homologs that cause sequestration of the guanine nucleotide exchange factor, preventing the wild-type factor from being activated. This complements the *in vitro* observation of an interfering effect of both these mutants on the stimulation by EF-Ts of the dissociation of EF-Tu[wt]•GDP (Krab & Parmeggiani, 1999a; Krab & Parmeggiani, 1999b). Especially mutant D80N has a very strong growth-inhibiting effect, indicating that the available EF-Ts is efficiently sequestered. The slow residual growth is probably mostly sustained by the intrinsic nucleotide exchange rate on EF-Tu. Coexpression of EF-Ts at a 1:1 stoichiometric ratio completely alleviates this growth inhibition. The fact that growth inhibition takes place shortly after the mutant EF-Tu is expressed testifies to the relatively fast folding of mutants T25A and D80N assisted by the residual guanine nucleotide binding activity, in contrast to mutant D138N, for which the folding lags so far behind expression that no noticeable growth inhibition takes place.

Recently several papers have shown that EF-Tu shows some chaperon-like properties *in vitro*, binding to and stimulating the refolding of denatured proteins (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998). This observation was subsequently extended also to the translation factors EF-G and IF-2 (Caldas *et al.*, 2000). Moreover, EF-Tu was shown to have quite reactive cysteines, lending it protein-disulfide isomerase activity, another important function in the process of establishing and maintaining correct protein structure (Richarme, 1998). While the relevance of these findings for protein folding *in vivo* is not clear and requires further investigation, we establish here that in certain conditions exchange factor EF-Ts can act as a steric chaperon for the folding of EF-Tu in the cell. Thus it is quite interesting that several protein factors that are essential for the protein production machinery in the cell may have an additional function in

assisting the correct folding of the products as well.

In conclusion one can say that in the cell, the ability of EF-Tu to rapidly fold into a native conformation greatly depends on its ability to bind nucleotide, acting as a template around which the correct structure of EF-Tu takes shape. When the affinity for nucleotide is strongly reduced, folding can still occur relatively rapidly, but the level of correctly folded and thus soluble protein in the cytoplasm is limited by the amount of available EF-Ts. When no cellular nucleotide can bind at all, the folding to a soluble native conformation hardly or only very slowly occurs. A native folding of EF-Tu can then only be achieved in the presence of equal amounts of EF-Ts. Thus EF-Ts acts as a steric chaperon for the folding of EF-Tu.

Chapter 7

Characterization of the action of Enacyloxin IIa, a new antibiotic that acts specifically on elongation factor Tu[‡]

Ivo M.Krab, Rengül Cetin, Pieter H.Anborgh, Robbert H.Cool, Toshihiko Watanabe, Takeyoshi Sugiyama, Kazuo Izaki and Andrea Parmeggiani

[‡]This chapter is based on the article by Cetin, Krab *et al.* that appeared in *EMBO J.* **15**, 2604-2611 (1996), taking into account the work of Zuurmond *et al.* in *J. Mol. Biol.* **294**, 627-637 (1999) that has revised the idea of a secondary direct action of enacyloxine IIa on the ribosome described in the original publication. This chapter also presents additional experimental work that underlines similarities and differences between the biochemical actions of enacyloxine IIa and that of kirromycin.

ABSTRACT

This work analyzes the action of enacyloxin IIa, an inhibitor of bacterial protein biosynthesis. Enacyloxin IIa (IC₅₀ on poly(Phe) synthesis: ~70 nM) is shown to affect intrinsic properties of EF-Tu like the interaction between elongation factor (EF) Tu and GTP or GDP. In particular, the dissociation of EF-Tu•GTP is strongly retarded, causing the *K_d* of EF-Tu•GTP to decrease from 500 to 0.7 nM. In its presence, the migration velocity of both GTP- and GDP-bound EF-Tu on native PAGE is increased. EF-Tu•GTP can still form a stable complex with aminoacyl-tRNA (aa-tRNA), but protection of aa-tRNA against spontaneous deacylation is reduced, even at high aa-tRNA concentration, showing that the EF-Tu•GTP orientation with respect to the 3'-end of aa-tRNA is modified. However, the EF-Tu-dependent binding of aa-tRNA to the ribosomal A-site is impaired only slightly by the antibiotic and the activity of the peptidyl-transferase center, as determined by puromycin reactivity, is not affected. In contrast, the C-terminal incorporation of Phe into poly(Phe)-tRNA bound to the P-site is inhibited. In some respects, the action of the antibiotic resembles that of kirromycin. For example, both antibiotics compete with EF-Ts for EF-Tu and both allow formation of a stable complex with aa-tRNA, in contrast to the antibiotics pulvomycin and GE 2270A. In strong contrast to kirromycin, however, enacyloxin IIa binds only transiently to EF-Tu and does not stimulate EF-Tu GTPase. The conformation that enacyloxin IIa induces in EF-Tu•GDP is also less "GTP-like" than that induced by kirromycin as indicated by its trypsin sensitivity and by EF-Tu•GDP mediated binding to the ribosome in the presence of antibiotic. This new antibiotic, that is structurally unrelated to the three previously known antibiotic families, extends our understanding of how agents can inhibit protein synthesis through their action on EF-Tu.

INTRODUCTION

Elongation factor (EF)-Tu, a model GTP-binding protein acting in protein synthesis as the carrier of aminoacyl-tRNA (aa-tRNA) to the mRNA-programmed ribosome (see: Miller & Weissbach, 1977; Bosch *et al.*, 1983; Weijland *et al.*, 1992; Krab & Parmeggiani, 1998), is the specific target of three different types of antibiotics: kirromycin (Wolf *et al.*, 1974; Parmeggiani & Swart, 1985), pulvomycin (Pingoud *et al.*, 1982a) and GE2270 A (Anborgh & Parmeggiani, 1991; Shimanaka *et al.*, 1995; Landini *et al.*, 1996). These drugs, whose chemical structures are very different, are inhibitors of bacterial protein biosynthesis *in vivo* and *in vitro*. The study of their action was useful in understanding the regulation of the EF-Tu encoding genes *tufA* and *tufB* (Bosch *et al.*, 1983; Vijgenboom & Bosch, 1989), the switch function and the catalytic activity of EF-Tu (see: Parmeggiani & Swart, 1985; Scarano *et al.*, 1995) and the structure-function relationships of the resistance to antibiotics (Mesters *et al.*, 1994b; Zeef *et al.*, 1994; Boon *et al.*, 1995). These three antibiotics each have specific differential effects on the GTP- and GDP-bound forms of EF-Tu, the two states determining the activity of the factor in the elongation cycle. In elongation, GTP-bound EF-Tu forms a complex with aa-tRNA that binds to the mRNA-programmed ribosome, a reaction associated with the hydrolysis of GTP. EF-Tu•GDP is released from the ribosome as a consequence of a dramatic decrease in the affinity for aa-tRNA and the ribosome (Miller & Weissbach, 1977). Kirromycin hinders this release of EF-Tu from the ribosome by inducing in EF-Tu•GDP a conformation more like that of EF-Tu•GTP, capable of binding to aa-tRNA and the ribosome (Parmeggiani & Swart, 1985). Pulvomycin and GE2270 A both negatively affect the interaction between EF-Tu•GTP and aa-tRNA by inducing an anomalous EF-Tu•GTP conformation (Anborgh & Parmeggiani, 1991; Anborgh & Parmeggiani, 1993).

In 1982, the first member of a new family of antibiotics, named enacyloxin, was isolated from the bacterial genus *Frateriia* sp. W-315 (Watanabe *et al.*, 1982; Watanabe *et al.*, 1994). One of the eight members of this family isolated so far, enacyloxin IIa, inhibits bacterial protein biosynthesis *in vitro* (Watanabe *et al.*, 1991). It is unique in being the first polyenic antibiotic reported to have a linear structure (Watanabe *et al.*, 1992, Figure 1). The molecule

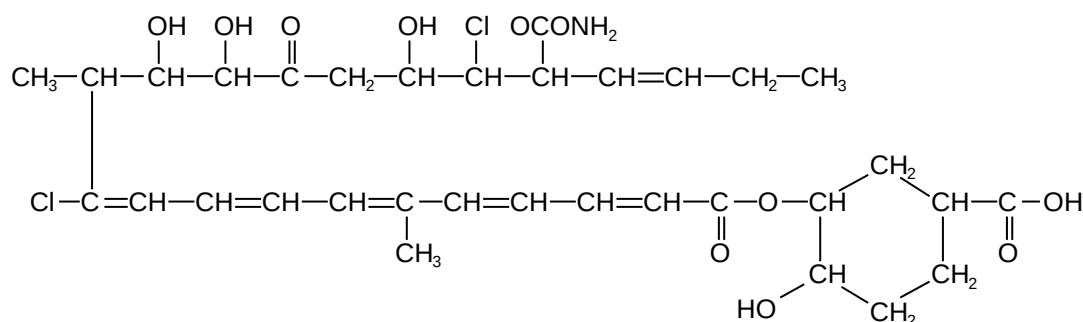


Fig. 1. Structure of enacyloxin IIa (Watanabe *et al.*, 1992).

(M_r 724) consists of a 3,4-dihydroxy-cyclohexanecarboxylic acid moiety and a linear polyoxygenated polyene carboxy structure with a carbamate group at C19'. Enacyloxin IIa has a bacteriostatic action on both Gram-positive and Gram-negative bacteria, and is slightly active against fungi but inactive against yeasts (Watanabe *et al.*, 1982).

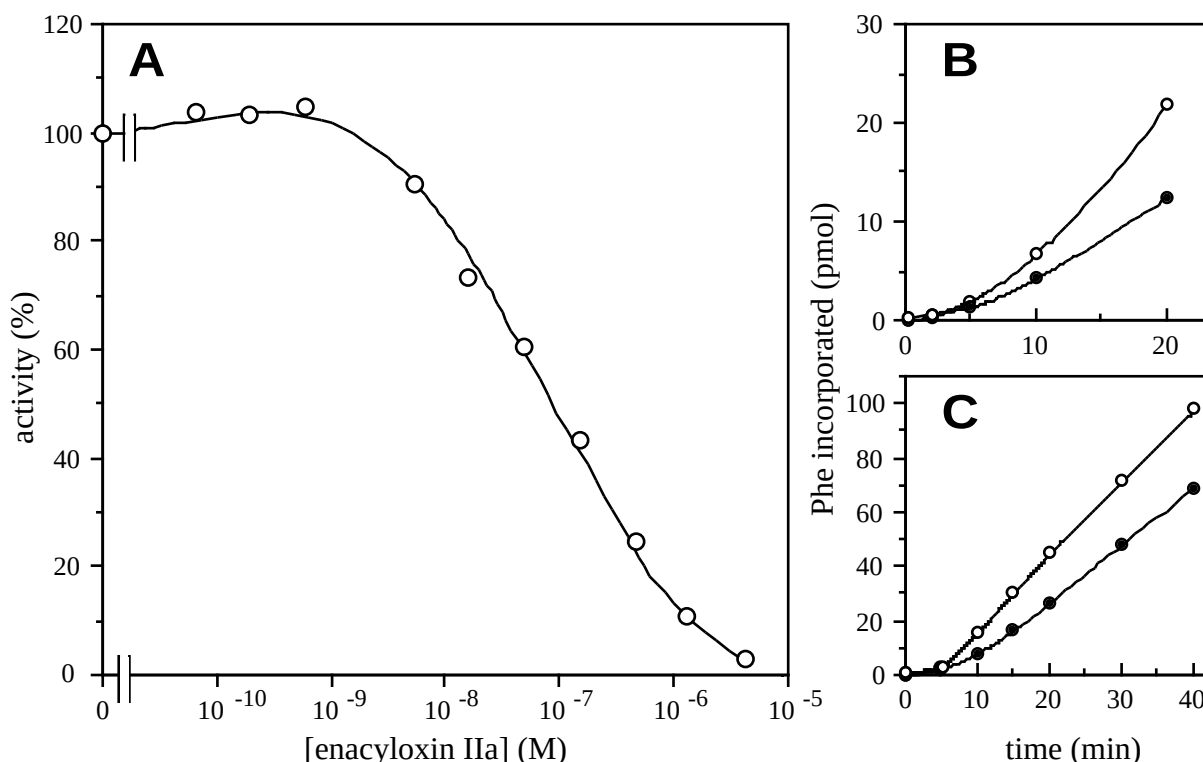


Fig. 2. Poly(U)-directed poly(Phe) synthesis, as a function of increasing concentration of enacyloxin IIa (A), and using EF-Tu•GTP pre-incubated with enacyloxin IIa in the absence (B) or in the presence of ribosomes (C). (A) The reaction mixture (30 μ l) contained 25 mM Tris-HCl, pH 7.6, 86 mM NH_4Cl , 6.7 mM MgCl_2 , 7 mM β -mercaptoethanol, 1 mM ATP, 0.25 mM GTP, 1 mM PEP, PK (40 μ g/ml), 0.7 μ M EF-Tu, 0.3 μ M EF-Ts, 0.15 μ M EF-G, 0.4 μ M ribosomes, poly(U) (80 μ g/ml), saturating amounts of Phe-tRNA synthetase, 165 μ g of bulk *E. coli* tRNA, 5 μ M [^3H]Phe (2900 d.p.m./pmol) and enacyloxin IIa at the indicated concentrations. After 10 min at 30°C, under conditions of linear Phe incorporation, 30 μ l of the reaction mixture were spotted on glass fibre filter discs (Whatmann GF/A) and the incorporated Phe was determined as hot TCA-insoluble material as reported (Anborgh & Parmeggiani, 1991). The results are expressed as a percentage of the activity obtained in the absence of the antibiotic. (B) EF-Tu•GTP (100 pmol), prepared as described in Materials and Methods, was incubated for 10 min at 0°C in 50 μ l of standard buffer in the presence (●) or in the absence (○) of 50 μ M enacyloxin IIa. The two mixtures were applied to small Sephadex G25 columns (10 x 0.7 cm) and 100 μ l fractions were collected. The two fractions containing EF-Tu were combined and 100 μ l of this solution were added to 100 μ l of a reaction mixture containing the same components as in (A). Samples (40 μ l) were withdrawn at the given times, spotted on glass fibre filters (Whatmann GF/A) and the poly(Phe) synthesized was determined. (C) EF-Tu•GTP (100 pmol) was incubated with 125 pmol of ribosomes for 10 min at 0°C in 50 μ l of standard buffer in the presence (●) or in the absence (○) of 50 μ M enacyloxin IIa. These two solutions were treated as in (B). The two fractions (200 μ l) eluted from the Sephadex G25 column containing EF-Tu and ribosomes were combined and added to 300 μ l of a reaction mixture containing the missing components to reconstitute a poly(Phe) synthesizing mixture as in (A) without additional antibiotic. Samples (70 μ l) were withdrawn at the given times, spotted on glass fibre filters (Whatmann GF/A) and the poly(Phe) synthesized was determined.

In this work we have studied the mechanism of action of enacyloxin IIa by analyzing stepwise the elongation process *in vitro*, in order to localize its specific target. Enacyloxin IIa was found to bind to EF-Tu, whose interaction with GTP, GDP and aa-tRNA is altered. We compare its action to that of other EF-Tu-specific antibiotics. It shows most similarity to kirromycin, yet it has unique features differentiating it from the other antibiotics acting on EF-Tu and offering new insights into how antibiotics can hinder protein biosynthesis through their action on EF-Tu.

RESULTS

The action of enacyloxin IIa on protein biosynthesis *in vitro*

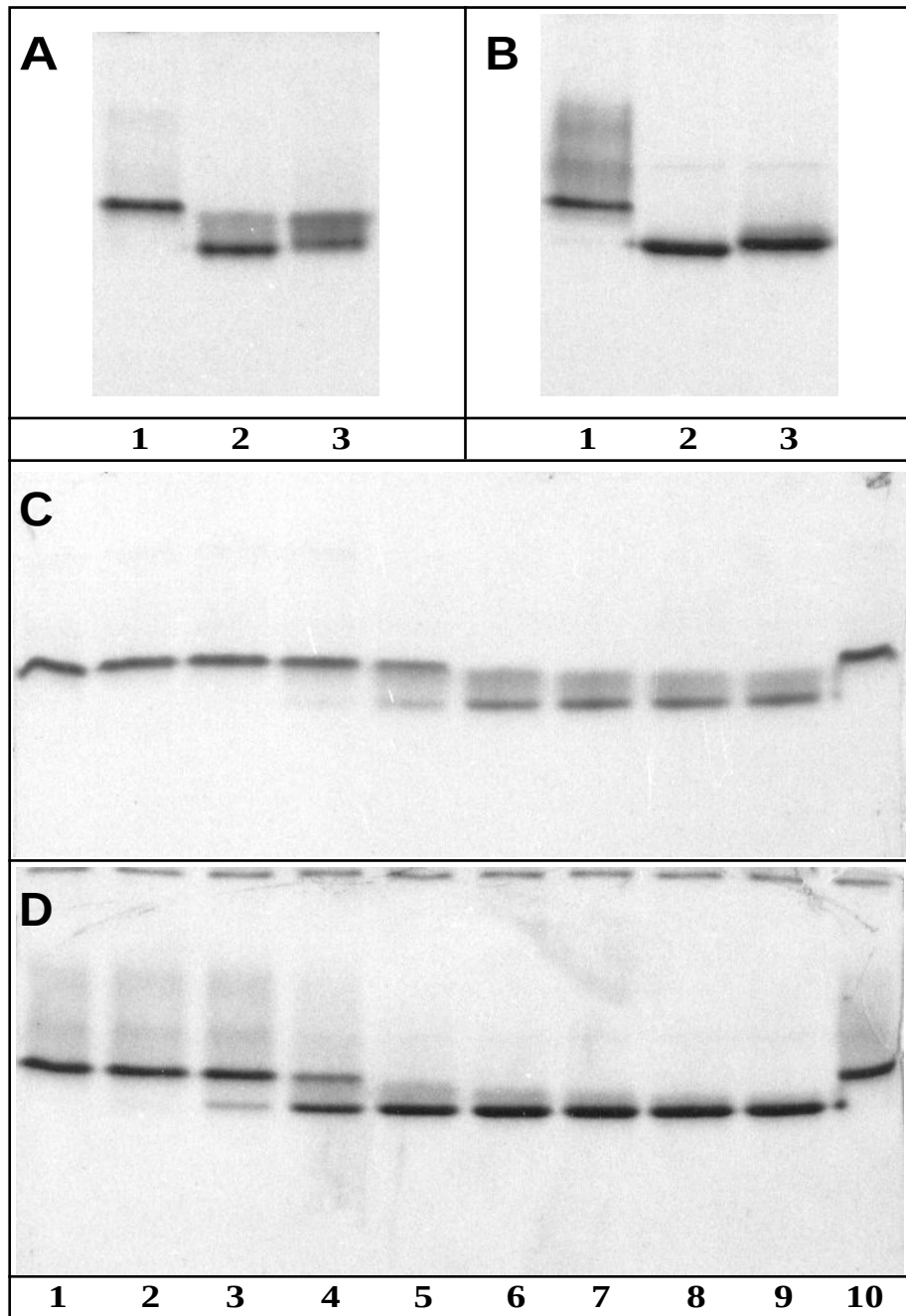
Enacyloxin IIa is a potent inhibitor of bacterial polypeptide synthesis *in vitro*. In a minimal system of purified components from *Escherichia coli*, poly(U)-dependent poly(Phe) synthesis was 50% inhibited at ~70 nM antibiotic (IC_{50}) (Figure 2A). We then proceeded to determine whether the antibiotic affects ribosomes or EF-Tu, the two most probable candidates for its inhibitory effect. For this we pre-incubated individual components (EF-Tu•GDP, EF-Tu•GTP, ribosomes or EF-Tu•GTP plus ribosomes) with enacyloxin IIa, after which non-stably bound excess antibiotic was removed by passing the mixtures through short Sephadex G25 columns (7 x 0.7 cm). Then a full poly(Phe) synthesis system was reconstituted by adding the missing components and phenylalanine incorporation was measured. Pre-treated EF-Tu•GDP or ribosomes were as active in poly(Phe) synthesis as untreated components (not shown), whereas pre-treated EF-Tu•GTP alone (Figure 2B) or in combination with ribosomes (Figure 2C) were 30-40% less active than the control. These results show that, under specific conditions, enacyloxin IIa can bind to EF-Tu•GTP and they prompted us to examine its effect on the intrinsic properties of the factor.

Enacyloxin IIa modifies the electrophoretic behaviour of EF-Tu

The electrophoretic migration of EF-Tu•GTP and EF-Tu•GDP on native PAGE is affected specifically by the binding of kirromycin, pulvomycin or GE-2270 A (Anborgh & Parmeggiani, 1991). As shown in Figure 3, enacyloxin II, too, can influence the electrophoretic pattern of EF-Tu•GDP (Figure 3A, third lane) and EF-Tu•GTP (Figure 3B, third lane). The migration velocity of both EF-Tu forms is increased, an effect resembling that of kirromycin (second lane in Figure 3A and 3B), though to a somewhat lesser extent. In the case of EF-Tu•GDP, one observes the presence of second component (cf. also Figure 3C), probably due to the dissociation of enacyloxin from the factor (Figure 3A, third lane). The extra bands in lane one of Figure 3B (EF-Tu•GTP) are due to aggregation phenomena especially evident in the absence of the antibiotic (cf. also the first lanes of Figure 3D). By following on the gel the effect of increasing concentrations of the antibiotics (from 2.5 and 160 μ M) we were

Fig. 3. Migration of EF-Tu•GDP (A and C) and EF-Tu•GTP (B and D) on native PAGE in the absence of in the presence of enacyloxin IIa or kirromycin (A and B) and as a function of the concentration of enacyloxin IIa (C and D). **(A)** EF-Tu•GDP (60 pmol) was incubated in 10 μ l of standard buffer in the absence (lane 1) or in the presence of 50 μ M kirromycin (lane 2) or enacyloxin IIa (lane 3) for 10 min at 0°C. **(B)** EF-Tu•GTP (60 pmol) in 10 μ l of standard buffer was incubated in the absence (lane 1) or in the presence of 50 μ M kirromycin (lane 2) or enacyloxin IIa (lane 3) for 10 min at 0°C. **(C)** EF-Tu•GDP (60 pmol) was incubated for 10 min at 0°C in 10 μ l of standard buffer in the absence (lane 1 and 10) or in the presence of 2.5, 5, 10, 20, 40, 80, 120 and 160 μ M (lanes 2-9, respectively) antibiotic. **(D)** EF-Tu•GTP (60 pmol) in 10 μ l of standard buffer was converted to EF-Tu•GTP and incubated for 10 min at 0°C in 10 μ l buffer in the absence of the antibiotic (lanes 1 and 10) or in the presence of 2.5, 5, 10, 20, 40, 80, 120 and 160 μ M (lanes 2-9, respectively). The

mixtures were applied to non-denaturing, discontinuous 12% PAGE at 8°C in a mini-gel apparatus using an electro-phoretic buffer containing Tris-glycine, pH 8.3, supplemented in the upper chamber with 20 μ M GDP in (A) and (C), 20 μ M GTP in (B) and (D).



able to estimate an apparent affinity of enacyloxin IIa for EF-Tu•GDP and EF-Tu•GTP according to this method (Figure 3C and D, respectively). The concentration of antibiotic causing 50% of the EF-Tu molecules to migrate at a higher velocity was ~6.5 μ M for EF-Tu•GTP and 30 μ M for EF-Tu•GDP.

Table I. Effect of enacyloxin IIa on the dissociation half lives, dissociation constants, dissociation and association rate constants of the EF-Tu•GTP and EF-Tu•GDP complexes at 0 °C

System	Dissociation half-life (min)	Dissociation rate const. $10^4 \times k_{-1}$ (/s)	Association rate const. $10^{-4} \times k_{+1}$ (/s•M)	Dissociation const. ^a K_d (nM)
EF-Tu•GDP	80	0.8	6.5	1.2 ^b
EF-Tu•GDP+enacyloxin IIa	17	6.2	7.8	8
EF-Tu•GTP	1	138	6.5	212
EF-Tu•GTP+enacyloxin IIa	40	2.8	39	0.7

^aThe dissociation constant was determined as $K_d = k_{-1}/k_{+1}$

^bValue accidentally reported as 2 in (Cetin *et al.*, 1996)

Enacyloxin IIa affects the interaction of EF-Tu with both GDP and GTP but not the intrinsic GTPase activity of EF-Tu

The activity of EF-Tu is controlled by GTP and GDP, the two ligands inducing the ‘on’ and ‘off’-conformation of EF-Tu, respectively. Both forms are altered by the three types of antibiotics acting on EF-Tu. Therefore, as the next step, we examined whether enacyloxin IIa also affected the dynamics of the EF-Tu•GDP and EF-Tu•GTP complexes. As summarized in Table I, enacyloxin IIa modifies all parameters of the interaction with GDP and GTP. It markedly retards the dissociation rate of the EF-Tu complex with GTP (50-fold) while moderately accelerating the association rate of this complex (6-fold) and the dissociation rate of EF-Tu•GDP (7- to 8-fold) (Table I). As a result of the enacyloxin IIa-induced modifications, the affinity of EF-Tu for GTP was increased several hundred fold (from 0.2 μ M to 0.7 nM), whereas the affinity for GDP was decreased 4-fold. In experiments not shown, the concentration of the antibiotic inducing half maximum retardation of the dissociation of EF-Tu•GTP corresponded to 70-90 nM.

The opposite effect of enacyloxin IIa on the dynamics of the EF-Tu interactions with GDP and GTP (the dissociation rate of the GDP complex is increased and that of the GTP complex decreased) causes a strong acceleration of the GDP to GTP exchange on EF-Tu (Figure 4). A similar effect has also been reported for kirromycin and pulvomycin (Fasano *et al.*, 1978; P.H. Anborgh, unpublished results).

We found enacyloxin IIa to be unable to measurably enhance the intrinsic GTPase of EF-Tu, in contrast to kirromycin, which markedly enhances this activity. A slight increase in the

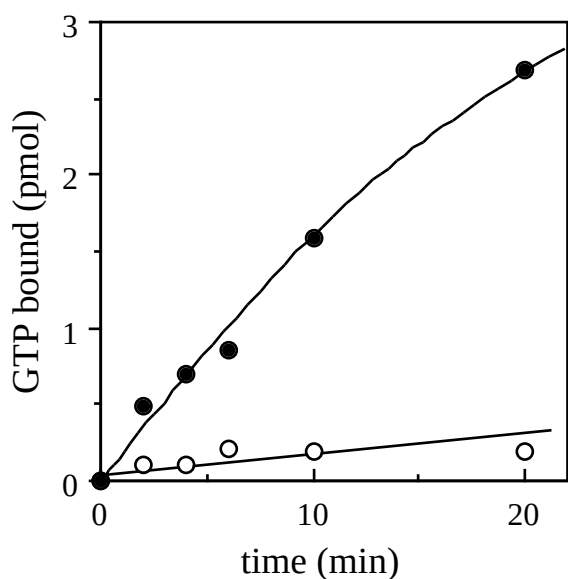


Fig. 4. Effect of enacyloxin IIa on the intrinsic GDP/GTP exchange of EF-Tu. The reaction mixture (140 μ l) contained 60 pmol of EF-Tu•GDP, 12 pmol of free GDP, 600 pmol of [γ - ^{32} P]GTP (800 d.p.m./pmol) and no (○) or 50 μ M enacyloxin IIa (●) in standard buffer at 0°C. The reaction was started by the addition of [γ - ^{32} P]GTP. At the indicated times, aliquots (20 μ l) were withdrawn and EF-Tu-bound radioactivity was determined by the nitrocellulose binding assay.

GTPase activity of EF-Tu by enacyloxin IIa became evident only in the presence of the ribosome (~20-30% higher than in the absence of the antibiotic) (not shown). Under similar conditions, kirromycin enhanced the EF-Tu GTPase activity >30-fold.

Enacyloxin IIa inhibits the stimulation of the EF-Tu GDP/GDP exchange reaction by EF-Ts

As mentioned in the preceding section, enacyloxin IIa causes an acceleration of the intrinsic exchange of the EF-Tu-bound GDP with free GTP. Even though its effect is much smaller than that induced by EF-Ts, the physiological exchange factor of EF-Tu, we exploited this property in determining whether enacyloxin IIa and EF-Ts compete for binding to EF-Tu. As shown in Figure 5, the presence of 10 μ M of the antibiotic abolished the strong stimulation by EF-Ts of the EF-Tu•GDP/GDP exchange, and induced nearly the same half-life as obtained in the absence of EF-Ts (cf. Table 1). By lowering the concentration of the antibiotic, the EF-Ts-dependent effect became progressively more evident. The amount of enacyloxin IIa inducing 50% of maximum retardation in the presence of EF-Ts was ~0.1 μ M. This apparent competition between antibiotic and EF-Ts for the binding to EF-Tu is characteristic also for kirromycin (Swart *et al.*, 1987; Anborgh *et al.*, 1991; Cetin *et al.*, 1998) but not for the other two antibiotics acting on EF-Tu.

Enacyloxin IIa modifies the interaction between EF-Tu and the aminoacylated 3'-terminal end of aminoacyl-tRNA

In the presence of enacyloxin IIa a stable complex between EF-Tu•GTP and aa-tRNA can still be formed, similarly to the action of kirromycin (Chinali *et al.*, 1977) and differently from pulvomycin and GE2270 A (Anborgh & Parmeggiani, 1991). In fact, on gel filtration

Fig. 5. Enacyloxin IIa competes with EF-Ts for binding to EF-Tu. EF-Tu•GDP (0.1 μ M) was incubated in 400 μ l standard buffer containing [^{3} H]GDP (50 pmol) (11 000 d.p.m./pmol) for 15 min at 30°C. After 5 min at 0°C in the presence of various concentration of enacyloxin IIa, EF-Ts (0.01 μ M) was added and the dissociation reaction started by the addition of a 1000-times excess of cold GDP at 0°C. At the indicated times, aliquots (40 μ l) were withdrawn and the EF-Tu-bound radioactivity determined by the nitrocellulose binding assay. c_0 represents the initial concentration of the EF-Tu•nucleotide complex and c_t the concentration at time t . EF-Tu plus EF-Ts in the presence of 0.125 ($\square$), 0.25 ($\bullet$), 0.5 (Δ), 2 ($\blacksquare$), 10 ($\circ$) and 50 ($\blacktriangle$) μ M enacyloxin IIa. Controls: without enacyloxin IIa and EF-Ts (+); without enacyloxin IIa plus EF-Ts ($\times$); with enacyloxin IIa minus EF-Ts ($\blacklozenge$).

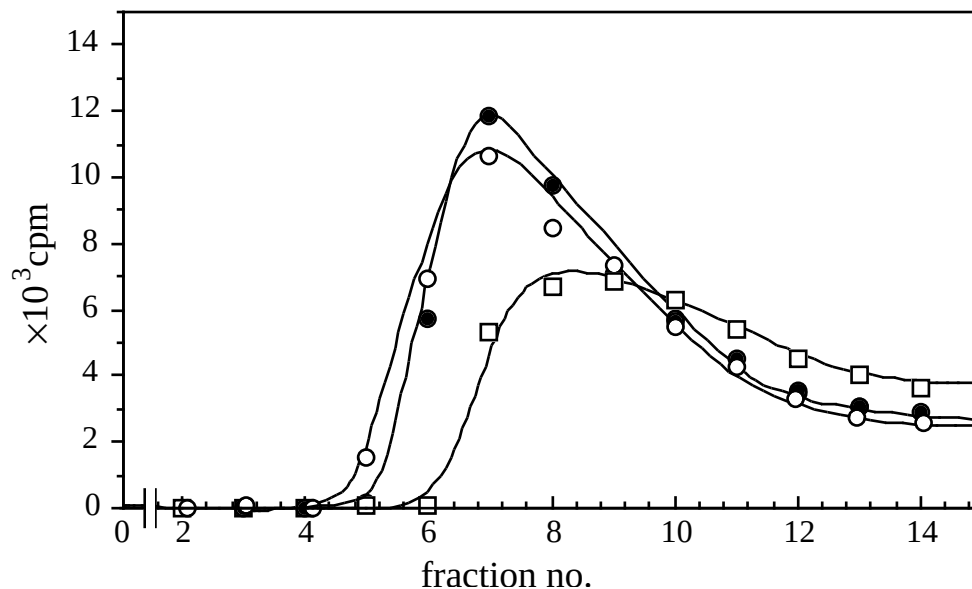
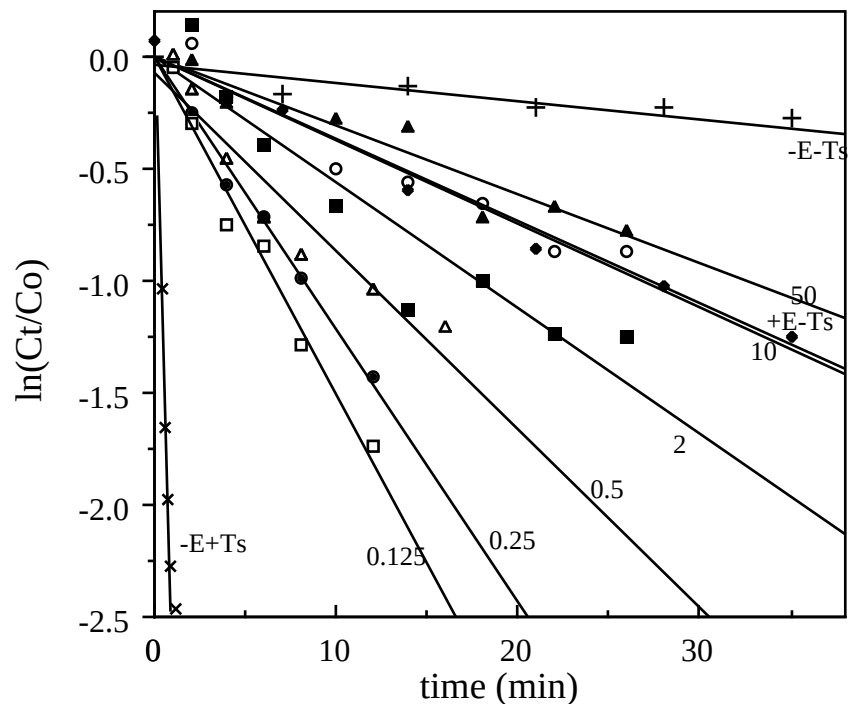


Fig. 6. Enacyloxin IIa does not affect the formation of the complex between EF-Tu•GTP and Phe-tRNA^{Phe}. The reaction mixtures contained in 50 μ l of standard buffer [^{14} C]Phe-tRNA^{Phe} alone ($\square$) or [^{14} C]Phe-tRNA^{Phe} (219 d.p.m./pmol) plus 260 pmol of EF-Tu•GTP with ($\bullet$) and without ($\circ$) 50 μ M enacyloxin IIa. After incubation (10 min at 0°C), they were passed over a Sephadex G-100 column (0.7 x 8 cm) and the radioactivity of the eluted fractions (100 μ l) measured. In the experiment with the antibiotic, the Sephadex G-100 column was equilibrated with 50 μ M enacyloxin IIa.

chromatography on Sephadex G-100 equilibrated with the antibiotic, EF-Tu•GTP•aa-tRNA showed virtually the same elution pattern as in the absence of enacyloxin IIa, both emerging earlier than aa-tRNA alone (Figure 6).

However, a characteristic property of the EF-Tu•GTP•aa-tRNA complex, was found to be different. Previously we showed that the protection of the Phe-tRNA^{Phe} aminoacyl ester bond against spontaneous hydrolysis by EF-Tu is practically absent in the presence of enacyloxin IIa (Cetin *et al.*, 1996). Because kirromycin is known to weaken the overall interaction of EF-Tu with aa-tRNA (Pingoud *et al.*, 1978; Abrahams *et al.*, 1991), we examined this further at higher concentrations of aa-tRNA, comparing with kirromycin (Figure 7). Before, at 0.5 μ M aa-tRNA we found virtually no protection, which is confirmed here by the very low protection at 0.63 μ M (Fig. 7A). At higher concentrations a protective effect does become visible, but even at 17 μ M no more than half of the protective effect is obtained. This is in contrast with kirromycin, which at the same concentrations protects better (Fig. 7B). Abrahams *et al.* measured an affinity between Phe-tRNA^{Phe} and EF-Tu•kirromycin from 2-6 μ M using various techniques. Thus at 17 μ M the EF-Tu should be close to saturated with Phe-tRNA^{Phe}. Nevertheless, even at this concentration it cannot protect the ester bond as well as in the absence

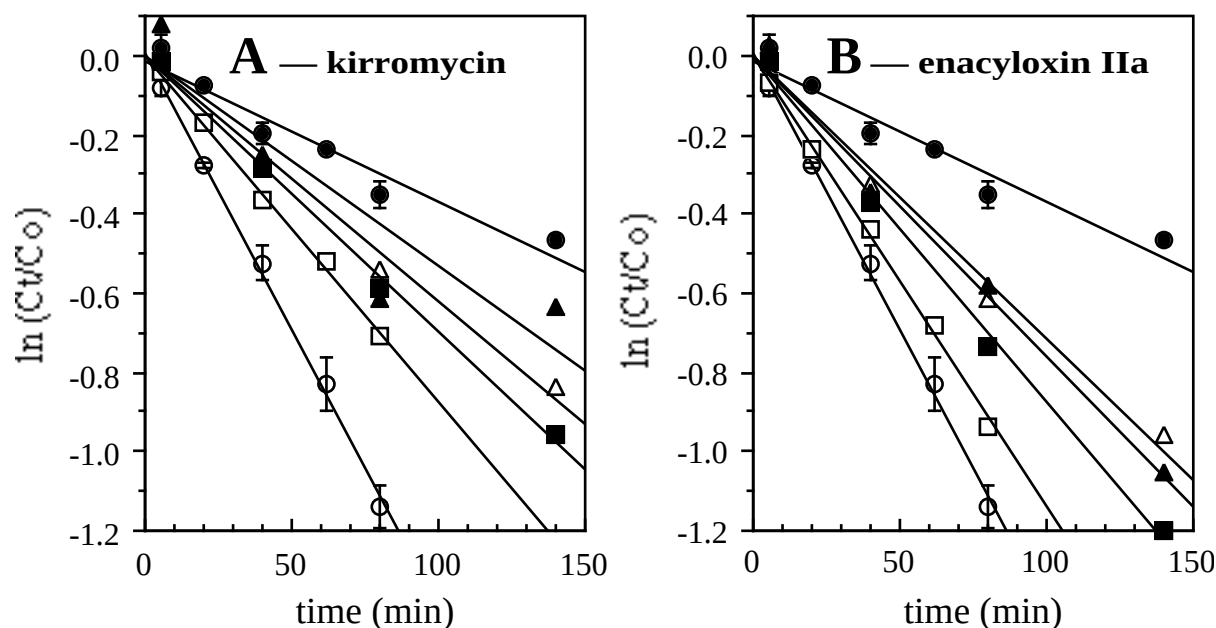


Fig. 7. Enacyloxin IIa and kirromycin reduce the protection by EF-Tu•GTP against the spontaneous deacylation of Phe-tRNA^{Phe}. Effect of kirromycin (A), or enacyloxin IIa (B), on the hydrolysis kinetics of [¹⁴C]Phe-tRNA^{Phe} (569 d.p.m./pmol), in the absence of EF-Tu (○), in its presence (●), or with additionally 100 μ M antibiotic (□, ■, △, ▲). Concentrations of aa-tRNA and EF-Tu were, respectively, 0.63 and 0.75 μ M (□); 1.9 and 2.25 μ M (■); 5.6 and 7.5 μ M (△); 17 and 22.5 μ M (▲). The controls in absence and presence of EF-Tu represent averages over all four concentrations. Reaction conditions were 25 mM Hepes-KOH, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 7 mM ME, and 0.05-2.5 mM GTP at 37 °C. The EF-Tu was converted to the GTP bound form by preincubating 15 min at 30 °C with 5 mM PEP and 30 μ g/ml pyruvate kinase. Samples (15 μ l) were withdrawn at the indicated times, spotted onto glass fibre filters, dipped into ico-cold 10% trichloroacetic acid, and the amount of [¹⁴C]Phe-charged tRNA^{Phe} was measured.

of the antibiotic, indicating an altered relationship of EF-Tu with the 3' end of the aa-tRNA. If we assume that a possible reduction in affinity for aa-tRNA by enacyloxin IIa is of the same order of magnitude, this antibiotic causes the 3' end of tRNA to be more exposed than with kirromycin.

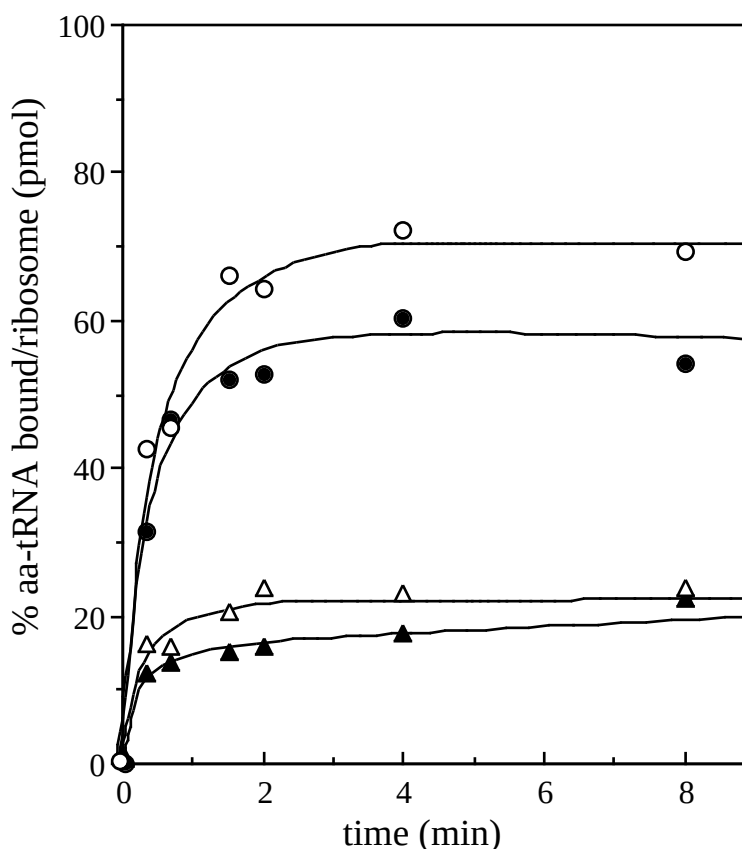
Despite the anomalous relationship with the aminoacylated 3'-terminal end of aa-tRNA, the EF-Tu-dependent binding of Phe-tRNA^{Phe} to the A site of poly(U)-programed ribosomes was only slightly affected by the antibiotic, as determined by following the kinetics of the binding at 30 °C (Figure 8) and 0°C (not shown). The maximal amount of Phe-tRNA^{Phe} bound to the ribosome was <20% decreased. The EF-Tu-independent binding of aa-tRNA only occasionally showed (as in the experiments of Figure 8) a slight inhibition. Experiments carried out in the presence of higher concentrations of the antibiotic (50 µM) gave similar results. No inhibition of the burst-like GTP hydrolysis associated with the enzymatic binding of aa-tRNA to the ribosomal A-site could be observed in the presence of enacyloxin IIa (not shown).

Enacyloxin IIa inhibits the incorporation of the amino acid into the polypeptide chain

As the next step, we investigated the influence of enacyloxin IIa on the incorporation of

Fig. 8. Influence of enacyloxin IIa on the binding of [¹⁴C]Phe-tRNA^{Phe} to the ribosomal A-site.

The final reaction mixture (165 µl) contained 25 mM Tris-HCl, pH 7.6, 86 mM NH₄Cl, 10 mM MgCl₂, 7 mM β-mercaptoethanol, 1 mM ATP, 0.25 mM GTP, 1 mM PEP, PK (40 µg/ml), no or 2.6 µM EF-Tu, 0.5 µM ribosomes, poly(U) (80 µg/ml), 0.5 µM tRNA^{Phe} (75% pure), 0.7 pmol [¹⁴C]Phe-tRNA^{Phe} (75% pure, 584 d.p.m./pmol), no or 10 µM enacyloxin IIa. EF-Tu•GTP and poly(U)•ribosomes had been preincubated with [¹⁴C]Phe-tRNA^{Phe} and tRNA^{Phe}, respectively, for 5 min at 30°C. The reaction was started by adding the tRNA^{Phe}•poly(U)•ribosome complex to EF-Tu•GTP and [¹⁴C]Phe-tRNA^{Phe}, and the extent of the binding of [¹⁴C]Phe-tRNA^{Phe} to the ribosomal A-site was determined at the indicated times on 25 µl samples. Phe-tRNA^{Phe} plus EF-Tu•GTP with (●) and without (○) enacyloxin IIa; Phe-tRNA^{Phe} minus EF-Tu•GTP with (▲) and without (Δ) enacyloxin IIa.



[^{14}C]Phe from [^{14}C]Phe-tRNA^{Phe} into the C-terminal end of poly(Phe) chains located in the P-site. For this purpose, ribosomal preparations were used that carry only poly(Phe) chains located in the P-site, as determined by the puromycin reaction (Wolf *et al.*, 1974). As illustrated in the experiments of Figure 9, carried out with ribosomes containing poly(Phe) chains only in the P-site (25% of the total ribosomal population present in the assay), enacyloxin IIa was found to inhibit the C-terminal incorporation of [^{14}C]Phe from enzymatically bound [^{14}C]Phe-tRNA^{Phe}. The IC₅₀ of enacyloxin IIa was $\sim 2\ \mu\text{M}$.

While we initially reported an inhibition of C-terminal incorporation by the antibiotic in the case of non-enzymatic binding of [^{14}C]Phe-tRNA^{Phe} to the ribosomal A-site as well (Cetin *et al.*, 1996), a contradictory finding was brought to our notice since (Zuurmond *et al.*, 1999a). When reexamining the matter we, too, were unable to reproduce (not shown) our previous result (obtained in several experiments). Likely our previous observation was due to an unexpected contamination of the reaction mixture by EF-Tu during separation over the Sepharose 4B column.

In order to rule out that enacyloxin IIa affects the peptidyl transferase reaction directly we also measured the reactivity of poly(Phe) chains located in the P-site to puromycin. This was virtually unaffected by the antibiotic: even in the presence of high concentrations of enacyloxine

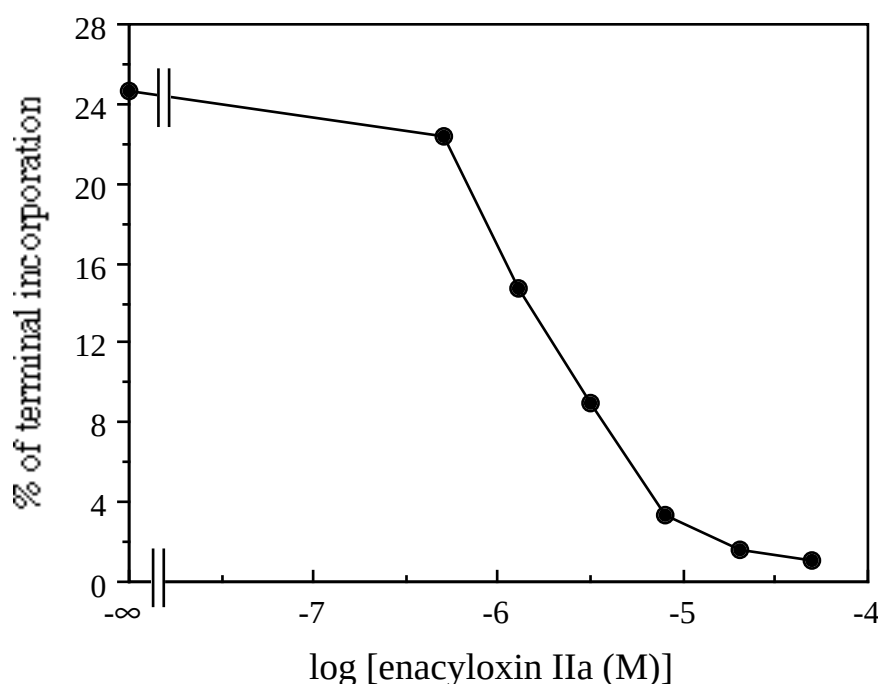


Fig. 9. Determination of the C-terminal incorporation of [^{14}C]phenylalanine into poly(Phe)-tRNA chain bound to the ribosomal P-site, as a function of the concentration of enacyloxin IIa. Ribosomes (28 pmol) carrying poly(Phe)-tRNA in the P-site, 44 pmol [^{14}C]Phe-tRNA^{Phe} (75% pure), 0.5 mM GTP, 1 mM PEP, PK (40 $\mu\text{g}/\text{ml}$) and enacyloxin IIa at the indicated concentrations were incubated for 30 min at 30°C in 186 μl standard buffer with 50 pmol EF-Tu•GTP. The incorporation of [^{14}C]phenylalanine into the poly(Phe)-tRNA was then determined as reported (see Materials and methods and: Wolf *et al.*, 1974)

Ia (50 μ M) ~90% of the ^{14} C-labeled poly(Phe) in the P-site could be transferred to puromycin.

EF-Tu is not strongly retained on the ribosome in the presence of enacyloxin IIa

We have also examined the possibility that enacyloxin IIa may retard the release of the EF-Tu•GDP complex from the ribosome, as in the case of kirromycin (Wolf *et al.*, 1974; Wolf *et al.*, 1977). For this purpose, we used gel filtration chromatography on short Sepharose 4B columns equilibrated with enacyloxin IIa, over which a poly(Phe) synthesizing mix triggered with [^{3} H]GTP•EF-Tu was passed. In the presence of the antibiotic no co-migration of the labeled nucleotide with the ribosomal peak was detected (not illustrated), as was found for kirromycin in a similar experiment (Wolf *et al.*, 1974; Wolf *et al.*, 1977). This suggests that after hydrolysis of the EF-Tu-bound [^{3} H]GTP, EF-Tu•[^{3} H]GDP leaves the ribosome. However, we cannot rule out that it is due to the dissociation of the nucleotide from the EF-Tu•enacyloxin IIa•Phe-tRNA^{Phe} complex, which remains bound. Interestingly, Zuurmond *et al.* (Zuurmond *et al.*, 1999a) found that enacyloxin IIa sensitivity is dominant over resistance, as for kirromycin (Fischer *et al.*, 1977; van de Klundert *et al.*, 1978), suggesting that enacyloxin IIa also causes “ribosome jam”. This apparent contradiction with our inability to detect a retention of EF-Tu•enacyloxin IIa on the ribosome prompted us to do some experiments that more closely compare specific effects of these two antibiotics. While we did attempt to also directly measure release of EF-Tu from the ribosome after enzymatic binding, technical difficulties prevented us from drawing conclusions about the release rates in the presence of either antibiotic (not shown).

EF-Tu•GDP•enacyloxin IIa can sustain enzymatic binding

The retention by kirromycin of EF-Tu on the ribosome after delivery of aa-tRNA is connected with the antibiotic’s property to induce a more “GTP-like” structure in EF-Tu•GDP, increasing its affinity for aa-tRNA. This allows even EF-Tu•GDP to deliver aa-tRNA to the ribosomal A-site. We therefore compared enzymatic binding of aa-tRNA by both EF-Tu•GTP and EF-Tu•GDP in the presence of enacyloxin IIa or kirromycin. Figure 10 B shows that kirromycin strongly stimulates enzymatic binding by EF-Tu•GDP in the absence of added monovalent salt. With increasing ammonium chloride concentrations this stimulation decreases monotonically. Enzymatic binding by EF-Tu•GTP in the presence of kirromycin shows a similar decrease of efficiency with increasing NH_4^+ concentration, in contrast to the native situation, where apart from a ~25% lower efficiency in the absence of monovalent salts, the binding level reached remains constant over the range examined (Figure 10 A). Enacyloxin IIa shows a hybrid behaviour. While it does allow EF-Tu•GDP to somewhat stimulate binding of Phe-tRNA to the ribosome, this is optimal at lower NH_4^+ concentrations (up to 30 mM) and drops down quite

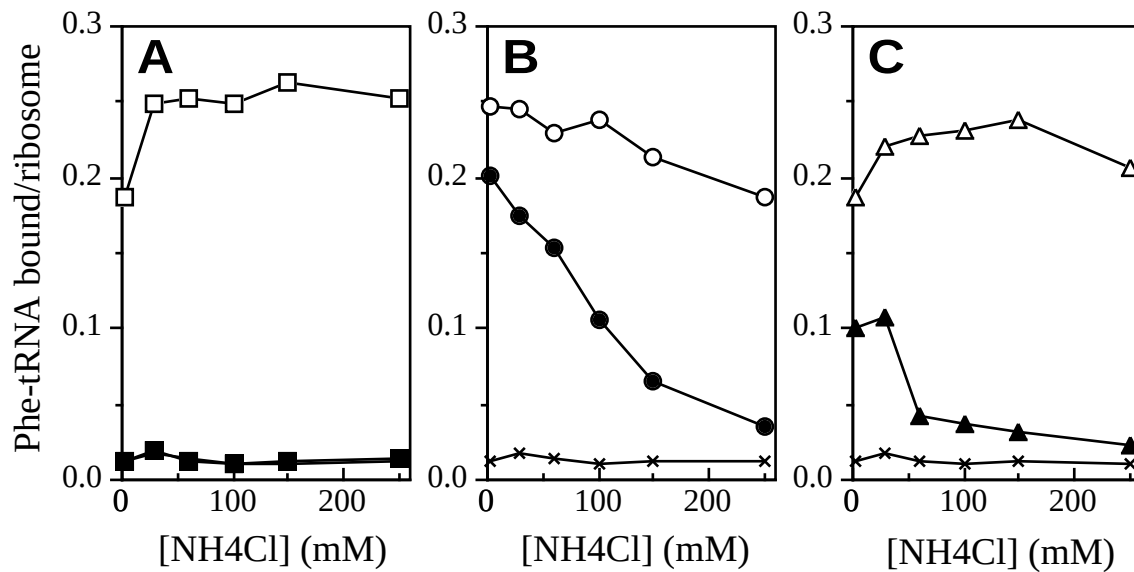


Fig. 10. Enzymatic binding of Phe-tRNA^{Phe} to the ribosomal A site by EF-Tu•GDP or EF-Tu•GTP in the presence of antibiotics as a function of NH₄Cl concentration. 45 μ l mix containing 22 pmol [¹⁴C]Phe-tRNA^{Phe} and 22.5 pmol EF-Tu•GTP ($\square$, $\circ$, Δ), EF-Tu•GDP ($\blacksquare$, $\bullet$, $\blacktriangle$) or no EF-Tu ($\times$) (1105 d.p.m./pmol), as well as no antibiotic (A), 50 μ M kirromycin (B) or 50 μ M enacyloxin IIa (C) were added to 5 μ l mix containing 10 pmol ribosomes preincubated with 1.5 μ g poly(U) and 10.5 pmol deacylated tRNA^{Phe} at 30 °C. After 3 min the amount of radioactivity present on ribosomes was determined by the nitrocellulose binding assay. Buffer conditions were 50 mM Tris-HCl, pH 7.6, the indicated concentration of NH₄Cl, 7 mM MgCl₂, 7 mM β -mercaptoethanol and 100 μ M GDP (closed symbols) or 100 μ M GTP, 1 mM phosphoenolpyruvate and 20 μ g/ml pyruvate kinase (open symbols).

strongly at higher concentrations (Figure 10 C). Also EF-Tu•GTP in the presence of this antibiotic shows a profile that is more similar to the native situation than to that in the presence of kirromycin.

Enacyloxin IIa enhances the trypsin digestion rate of EF-Tu•GDP more than kirromycin

A direct structural probe for the conformation of EF-Tu is provided by its sensitivity to trypsin, which has a major cleavage site after arginine 58, a residue of the effector region that shows dramatic conformational differences between the GDP- and GTP-bound forms of EF-Tu. As shown in Figure 11, kirromycin increases the rate of trypsin digestion more than twice, while enacyloxin IIa causes an intermediate stimulation.

The lability of the binding of enacyloxin IIa is increased by complexation with aa-tRNA

We have already seen in Figures 2 B and C that gel filtration of enacyloxin IIa-treated EF-Tu can at least partially restore its activity in poly(Phe) synthesis. This is in sharp contrast to kirromycin, which can only be removed from the factor by employing competition for binding

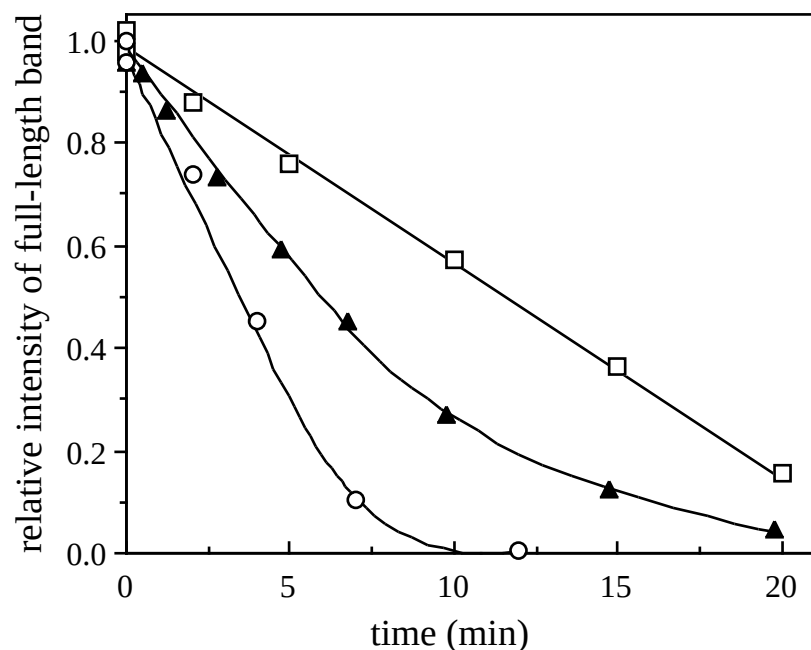
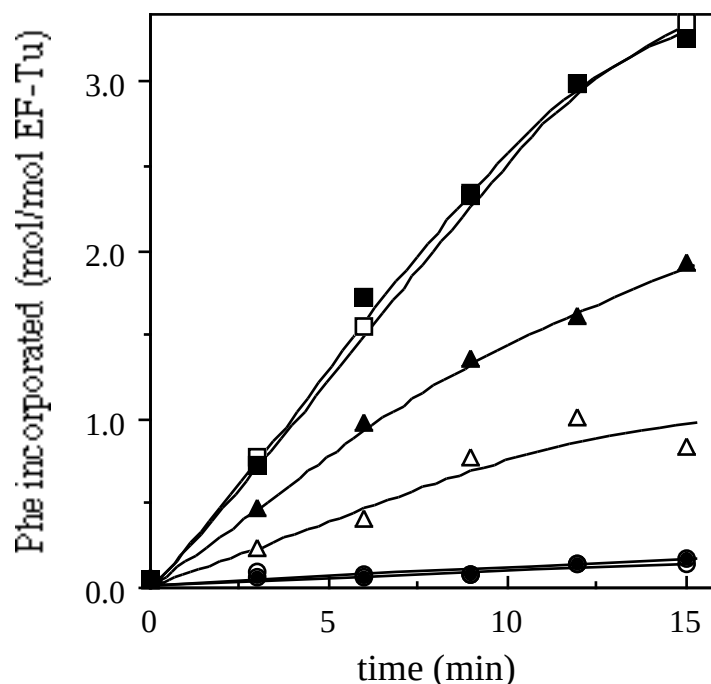


Fig. 11. Tryptic digestion of EF-Tu in the presence of antibiotics. 40 $\mu\text{g/ml}$ EF-Tu was digested with 5 $\mu\text{g/ml}$ trypsin in 50 mM Tris-HCl pH 7.5, 5 mM MgCl_2 , 1 mM EDTA, 2.5 mM CaCl_2 , and no antibiotic ($\square$), 50 μM kirromycin ($\circ$) or 50 μM enacyloxin IIa ($\blacktriangle$). At the indicated times 5 μl samples were taken and mixed with 5 μl 0.5 mg/ml trypsin inhibitor to stop the reaction. Samples were then separated on SDS-PAAGE, stained with Coomassie Brilliant Blue and the dried gels scanned.

with EF-Ts (Swart *et al.*, 1987; Anborgh & Parmeggiani, 1991). Because in the cell much of EF-Tu is present in the form of a complex with aa-tRNA, we examined this point further by comparing the poly(Phe) synthesizing ability of both EF-Tu•GTP and its complex with aa-tRNA preincubated with enacyloxin IIa or kirromycin and then passed through G50 sephadex

Fig. 12. Poly(Phe) synthesis with EF-Tu and its aa-tRNA complex preincubated with antibiotics. 150 pmol EF-Tu•GTP (open symbols) or 150 pmol EF-Tu•GTP plus 150 pmol [^{14}C]Phe-tRNA^{Phe} were incubated without antibiotics ($\square$, $\blacksquare$) with 50 μM kirromycin ($\circ$, $\bullet$) or with enacyloxin IIa (Δ , $\blacktriangle$) in 35 μl standard buffer on ice, then passed through small G50 Sephadex columns. The 200 μl eluate containing EF-Tu was collected and 45 of the solution added to 15 μl mix (preincubated for 15 min at 30 $^\circ\text{C}$) containing the other components necessary to reconstitute a poly(Phe) synthesis system with final conditions of 25 mM Hepes pH 7.6, 70 mM NH_4Cl , 7 mM MgCl_2 , 7 mM β -mercaptoethanol, 1.5 mM ATP, 0.5 mM GTP, 3.8 mM phosphoenolpyruvate, 30 $\mu\text{g/ml}$ pyruvate kinase, 1.5 μM ribosomes, 50 $\mu\text{g/ml}$ poly(U), 0.8 μM EF-G, 0.05 μM EF-Ts, 3 μM tRNA^{Phe}, 20 μM [^{14}C]Phe (160 d.p.m./pmol) and 25 nM Phe-tRNA synthetase. At the indicated times during incubation at 30 $^\circ\text{C}$ 10 μl samples were withdrawn, spotted on glass fibre filters (Whatmann GF/A) and the poly(Phe) synthesized was determined.



columns (Figure 12). Not surprisingly, the kirromycin-preincubated factor was in both cases unable to sustain poly(Phe) synthesis. In the case of enacyloxin IIa preincubation the complex with Phe-tRNA regained twice as much activity as EF-Tu•GTP alone, indicating that the antibiotic is more readily released from the aa-tRNA complex.

Does enacyloxin IIa act on other steps of the elongation cycle?

We tested whether enacyloxin IIa could influence the translocation of the polypeptide chain from the ribosomal A-site to the P-site. No measurable effect on the puromycin reactivity of ribosomes carrying poly(Phe) chains in the A- and P-sites upon addition of EF-G plus GTP was detected, nor was the ribosomal EF-G-dependent GTPase activity influenced by the presence of the antibiotic.

DISCUSSION

This work examines how enacyloxin IIa inhibits protein synthesis through its effect on EF-Tu. Like the other antibiotics acting on EF-Tu, enacyloxin IIa alters the intrinsic properties of the factor concerning nucleotide interaction, electrophoretic mobility in native gel and interaction with aa-tRNA. Moreover, we found that it inhibits the incorporation into the P-site bound polypeptide chain of aa-tRNA bound by EF-Tu to the ribosomal A-site. On the other hand it hardly inhibits the enzymatic (EF-Tu-mediated) binding of aa-tRNA to the A-site. Clearly the inhibition of C-terminal incorporation is due to an effect after binding. The peptidyl transferase center itself is not affected since enacyloxin IIa cannot prevent the puromycin reaction of preformed peptidyl chains in the P-site. Remains the possibility of a misorientation of the bound aa-tRNA in the ribosomal A site preventing peptidyl transferase. Alternatively, the antibiotic could cause a slow-down of the release of EF-Tu after GTP hydrolysis, thus blocking the peptidyl transferase reaction, as happens with kirromycin (Wolf *et al.*, 1974; Wolf *et al.*, 1977). However, we were unable to detect retention of the factor (through a labeled GTP) on the ribosome using the same method that shows this with kirromycin. This may appear in contradiction with a recent finding by (Zuurmond *et al.*, 1999a), that in a mixed EF-Tu population enacyloxin IIa sensitivity is dominant over resistance, like for kirromycin (Fischer *et al.*, 1977; van de Klundert *et al.*, 1978). The accepted explanation for the effect of kirromycin is the occurrence of a “ribosome jam” on the mRNA, where one stalled ribosome blocked by a sensitive EF-Tu on the polysome stops synthesis also on ribosomes following behind, even when they are served by resistant EF-Tu’s. It should however not be pictured that this is due to an irreversible binding of EF-Tu•GDP•kirromycin•aa-tRNA to the ribosome, but rather to a low release rate. On the experimental time-scale, the complex is held long enough that, after Sepharose 4B gel filtration, binding can still be detected. In the case of enacyloxin IIa

release may occur faster so the complex escapes detection with this method. Still, release may be slow enough to cause a “ribosome jam” on ribosomes elongating at high speed. Our attempts to measure the actual kinetics of release were unsuccessful, though indeed release of EF-Tu in the presence of either antibiotic can be seen (not shown). The inhibition of C-terminal incorporation of non-enzymatically bound aa-tRNA that we reported before could not be confirmed after reexamination. Thus EF-Tu appears to be not just the main target for inhibition by this antibiotic as we stated, but the sole one.

In many ways, the effects of enacyloxin IIa are more similar to kirromycin than to pulvomycin or GE2270 A. Like kirromycin, enacyloxin IIa competes with EF-Ts for binding to EF-Tu and, like kirromycin, it does not inhibit the formation of a stable ternary complex with aa-tRNA as do pulvomycin and GE2270 A (Wolf *et al.*, 1978; Anborgh & Parmeggiani, 1991; Anborgh & Parmeggiani, 1993). Moreover, several EF-Tu mutants resistant to kirromycin are also resistant to enacyloxin IIa, although the relative sensitivities differ between the two antibiotics (Zuurmond *et al.*, 1999a). On the other hand, a very characteristic effect of kirromycin, that of its stimulation of both intrinsic and ribosome-elicited GTPase on EF-Tu, is absent or very much reduced, respectively, with enacyloxin IIa. Both enacyloxin IIa and kirromycin change the interaction of EF-Tu•GTP and the aminoacylated 3'-terminal end of aa-tRNA. Kirromycin is known to reduce the affinity for aa-tRNA (K_d 2-6 μ M: Abrahams *et al.*, 1991), but even at 17 μ M Phe-tRNA^{Phe}, where EF-Tu should be almost saturated, it does not protect as fully as in the absence of antibiotic (Fig. 7). While we did not measure the affinity for aa-tRNA for EF-Tu•GTP•enacyloxin IIa, at any concentration the protective effect of complex formation on the ester bond is lower even than in the presence of kirromycin. At sub- μ M concentrations it is even virtually absent, as was published before (Cetin *et al.*, 1996). This suggests that in the presence of enacyloxin IIa the 3'-aa end is more exposed in the ternary complex than is the case in the presence of kirromycin. Also in other ways enacyloxin IIa influences EF-Tu differently from kirromycin. Kirromycin induces on EF-Tu•GDP a more “GTP-like” conformation (Chinali *et al.*, 1977). As a consequence EF-Tu•GDP•kirromycin has an increased affinity for aa-tRNA and is able to stimulate enzymatic binding of aa-tRNA to the ribosomal A-site, especially in the absence of monovalent cations (Figure 10). EF-Tu•GDP•enacyloxin IIa on the other hand is much less efficient at stimulating this reaction and shows an equivalent level at 0 and 30 mM salt concentration, which is more reminiscent of the factor in the absence of antibiotic. Also for enzymatic binding by EF-Tu•GTP we see a monovalent cation profile that is closer to the native situation with enacyloxin IIa than with kirromycin. A further probe for the structure of EF-Tu is provided by its sensitivity to trypsin cleavage. Trypsin preferentially cleaves EF-Tu after Arg-58, a site exposed on α -helix 1” in the GTP-form and at the tip of a hairpin loop touching domain 3 inside the funnel-like aperture in the GDP-bound form. As shown in figure 11, the kinetics of digestion in the presence of enacyloxin IIa is intermediary

between that in the presence of kirromycin and that in the absence of antibiotic. These experiments indicate that enacyloxin IIa causes a less pronounced structural alteration of EF-Tu than kirromycin does.

A characteristic that differentiates enacyloxin IIa from all other antibiotics that act on EF-Tu is the ease with which it dissociates from the factor. Removal of pulvomycin and GE2270 A bound to EF-Tu•GDP cannot be achieved, even after repeated passages through long filtration columns or after several days of dialysis (P.H. Anborgh, unpublished observations), and kirromycin is known to be released from EF-Tu only by exploiting the competition between the antibiotic and EF-Ts for binding to EF-Tu. In contrast, the interaction of enacyloxin IIa with EF-Tu or the ribosome is transient. In fact, dialysis or gel filtration chromatography can remove the antibiotic even from EF-Tu•GTP, to which the antibiotic binds more tightly than to EF-Tu•GDP, and leads to the reactivation of the factor in poly(Phe) synthesis. Binding of aa-tRNA facilitates this dissociation (Figure 12), which is likely a factor why in the presence of enacyloxin IIa EF-Tu can more easily be released from the ribosomal A site after delivery of the aa-tRNA than with kirromycin, thus providing one possible reason why we failed to detect a stalled ribosome complex.

Recently the first crystal structure of an antibiotic-EF-Tu complex, that of EF-Tu•GDP•GE2270 A, was determined (Heffron & Jurnak, 2000). In line with its property to interfere with aa-tRNA interaction and with the position of resistance mutations, GE2270 A binds to domain 2 in a position which in the GTP form would place it in the interface with domain 1 where the aminoacyl end of the ternary complex is accommodated (Nissen *et al.*, 1995). Because it also interferes with aa-tRNA binding and resistance mutations are located in domain 2, pulvomycin is likely to bind in the same general region. However, unlike for kirromycin and enacyloxin IIa, pulvomycin resistant mutations display GE2270 A sensitive behaviour (Zeef *et al.*, 1994). Kirromycin binding occurs in a different region since it has been shown to bind to EF-Tu simultaneously with GE2270 A (Landini *et al.*, 1996) or with pulvomycin (Pingoud *et al.*, 1982a). Indeed, since both kirromycin and enacyloxin IIa do not interfere with aa-tRNA binding like the other two antibiotics, they both compete with EF-Ts for binding, and the mutations inducing resistance to kirromycin/enacyloxin IIa line the interface between domains 1 and 3, they are likely to bind to similar (overlapping) sites near this interface (Cetin *et al.*, 1998; Krab & Parmeggiani, 1998). However, since enacyloxin IIa reduces protection of the aa-tRNA ester bond more than kirromycin does, the structural alterations induced by the binding of enacyloxin IIa may extend towards the domain 1-2 interface where the tRNA-bound amino acid is normally harboured. Also the fact that kirromycin stimulates EF-Tu GTPase while enacyloxin IIa does not, and the other differences noted above concerning the EF-Tu•antibiotic complex conformation, suggests divergences in the location and mode of their binding.

The influence of enacyloxin IIa on nucleotide interaction has the same general characteristics as that of the other EF-Tu-inhibiting antibiotics, resumed in Table II. Especially evident are the strong retardation of GTP release by 35 times or more for all antibiotics. The interaction parameters with GDP are in general less affected; in the case of GE 2270A there is even no effect at all. Overall, the modified parameters cause an increase in the spontaneous GDP to GTP exchange reaction, an effect physiologically mediated by the combined action of EF-Ts and aa-tRNA. While the antibiotics change the equilibrium in favour of the active (GTP-)form of EF-Tu, they cannot by themselves provide the acceleration of exchange necessary in physiological conditions. In this context it is interesting to note, that the concentration of the enacyloxin IIa inducing a 50% inhibition of C-terminal incorporation (IC_{50}) is approximately 2 μ M, while the IC_{50} in poly(U)-directed poly(Phe) synthesis is only 70 nM, $\sim$ 30 times less and lower than the EF-Tu concentration. This could be explained by a “ribosome jam” type of inhibition mechanism. On the other hand, also the measured IC_{50} of intrinsic EF-Tu properties, like the retardation of the dissociation of the EF-Tu•GTP complex, as well as the IC_{50} on the action of EF-Ts, are on the order of 0.1 μ M. This is much lower than the IC_{50} of inhibition of C-terminal incorporation, and similar to that of poly(Phe) synthesis, suggesting that the anomalous EF-Tu interaction with GDP/GTP and the competition between EF-Ts and enacyloxin IIa for binding to EF-Tu also play a role in the inhibitory action of the antibiotic on protein biosynthesis. In particular, the competition between enacyloxin IIa and EF-Ts for EF-Tu hinders the regeneration of the active form of EF-Tu, thus limiting the rate of polypeptide synthesis. This could mean that the inhibition of protein biosynthesis by enacyloxin IIa is a dynamic process. Ribosome stalling, the occurrence of which is suggested by the dominance of sensitivity over resistance, is only a part of that. It is also worth mentioning that the action of EF-Tu in protein biosynthesis is probably not limited to the proper positioning of aa-tRNA in the A-site. It appears to be involved in other as yet badly defined functions as well, as suggested by the existence of cooperative intermolecular phenomena of EF-Tu and by the hydrolysis of a second nucleoside triphosphate molecule per elongation cycle on EF-Tu during poly(Phe) synthesis (Ehrenberg *et al.*, 1990; Weijland & Parmeggiani, 1993; Bosch *et al.*, 1996).

In conclusion, we have characterized the action of a new antibiotic that specifically alters the properties of EF-Tu and is structurally unrelated to the other antibiotics that have EF-Tu as their target. With regard to its mode of action enacyloxin IIa seems to belong with kirromycin to one functional subclass of EF-Tu targeting antibiotics, while pulvomycin and GE2270 A form another. Nevertheless it has unique properties setting it apart from kirromycin and offering new insights into the way antibiotics can inhibit translation.

MATERIALS AND METHODS

Biological components

Purified EF-Tu, EF-Ts and EF-G, Phe-tRNA synthetase and NH_4Cl -washed ribosomes were prepared from *E. coli* as reported (Parmeggiani & Sander, 1981). tRNA^{Phe} from bulk tRNA or 70-80% pure from Sigma was used. Highly purified enacyloxin IIa, kept under argon, was dissolved in 100% methanol at a 10 mM concentration and stored at -30°C. The activity of this solution was stable for at least several weeks. Kirromycin, obtained as mocimycin from Dr. R. Beukers (Gist-Brocades) was used as a stable 5 mM mother solution in dimethylformamide and kept at -30°C (Wolf *et al.*, 1974). In the final solution for the various assays, the organic solvents carried over with the antibiotics and glycerol from EF-Tu and ribosomes never exceeded a 2% concentration and were also added to the controls.

Methods

Poly(U)-directed poly(Phe) synthesis was assayed by determining the radioactivity incorporated into hot trichloroacetic acid (TCA)-insoluble material (Swart *et al.*, 1987). C-terminal incorporation of [¹⁴C]Phe into poly(Phe) chains was determined, using ribosomes carrying poly(Phe)-tRNA^{Phe} only in the P-site. These ribosome complexes were prepared carrying out poly(U)-directed poly(Phe) synthesis to completion in the presence of a 20-fold excess of unlabeled Phe-tRNA^{Phe} (Wolf *et al.*, 1974). Under these conditions, all poly(Phe)-tRNA^{Phe} was bound to the ribosomal P-site, as confirmed by the inability of EF-G plus GTP to enhance the reactivity towards puromycin. Dissociation constants, and association and dissociation rate constants of the EF-Tu complexes with GDP or GTP and binding of Phe-tRNA^{Phe} to the ribosomal A-site were measured by the nitrocellulose binding assay; peptide bond formation, determined as puromycin reactivity, and Phe-tRNA charging, assayed by measuring the radioactivity incorporated into cold TCA-insoluble material, were carried out as described (Wolf *et al.*, 1974; Chinali *et al.*, 1977; Wolf *et al.*, 1977; Fasano *et al.*, 1978; Swart *et al.*, 1987; Anborgh & Parmeggiani, 1991). EF-Tu•GTP was prepared from 0.1-0.6 nmol of EF-Tu•GDP by pre-incubation (15 min at 30°C) with 3 mM phosphoenolpyruvate (PEP), 5 µg pyruvate kinase (PK) and 0.5 mM GTP in standard buffer. The GTPase activity of EF-Tu was measured as liberation of [γ -³²P]P_i from [γ -³²P]GTP, using the charcoal method (Parlato *et al.*, 1981). The standard assay buffer contained 50 mM Tris-HCl, pH 7.6, 60 mM NH_4Cl , 10 mM MgCl_2 , and 1 mM dithiothreitol. For other technical details, see legends to figures.

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Chapter 8

Summarising Discussion

While recent years have seen a tremendous progress in our knowledge of EF-Tu on the structural level, several functional questions could not be unambiguously answered from the newly available structures, such as the functional elements involved in the GTPase activity, the reason for the difference in affinity of EF-Tu for GTP and GDP and very importantly, its mode of interaction with the ribosome. The work presented in this thesis addresses such functional questions, attempting to correlate the experimental results with structural data. *Chapters 3-6* are devoted to the characterization of EF-Tu mutated in specific residues which were suspected to be involved in the GTPase activity of the factor. The properties of these mutants yield insight not only into EF-Tu's enzymatic function, but also into the way it interacts with the nucleotide and the exchange factor EF-Ts, as well as the folding of the factor in the cell. *Chapter 7* describes the characterization of the first member of a new antibiotic family that acts on EF-Tu. Antibiotics have been important tools in exploring the functioning of EF-Tu, and this new antibiotic, that shares some characteristics with kirromycin but displays some specific differences too, offers the opportunity to deepen our understanding of this center-stage player of the protein biosynthesis machinery.

The GTPase activity of EF-Tu — The general base hypothesis

GTP hydrolysis by EF-Tu is known since 1982 to take place via an S_N2 attack of a water nucleophile on the γ -phosphate, without protein intermediates (Eccleston & Webb, 1982). For this reaction mechanism catalysis can be achieved by i) increasing the nucleophilicity of the attacking group, ii) stabilizing the transition state, or iii) by increasing the efficiency (electrophilicity) of the leaving group. The first mechanism can occur either by specific base catalysis —ie. hydroxyl ion— or by general base catalysis involving a proton-abstracting molecule or residue of the protein. When the high-resolution structure of ras p21 was published in 1990 (Pai *et al.*, 1990) the hypothesis was put forward that switch II region residue Gln-61 could serve as a general base catalyst, activating a water molecule located at the γ -phosphate of the GTP for nucleophilic attack. Swart (Swart, 1987) hypothesized —on the basis of the structure of a preliminary determination of the G-domain of EF-Tu•GDP by the Århus group— that in EF-Tu the homologous residue His-84 could play a catalytic role, in conjunction with Glu-117 as the final proton acceptor. However, mutating Glu-117 to glutamine in the isolated EF-Tu G-domain did not change the GTPase activity (Harmark *et al.*, 1990), while substituting His-84 by glycine strongly reduced the intrinsic GTPase activity, but did not completely abolish it as expected in the case of a catalytic residue (Cool & Parmeggiani, 1991). The EF-Tu•GDPNP structure later showed His-84¹ to be closer to the γ -phosphate than in the GDP structure, however to be prevented from accessing the γ -phosphate by a barrier consisting of hydrophobic residues Val-20 and Ile-60; moreover, Glu-117 turned out to be away on the side (Bensch *et al.*, 1991; Kjeldgaard *et al.*, 1993). In *chapter 5* the functional properties were

¹ In this discussion the residue numbering of *E.coli* is used, even when referring to the homologous residues in the factor of *T. thermophilus* or *aquaticus*.

examined of full-length *E. coli* EF-Tu with substitutions of His-84 to either Ala, that cannot act as a base at all), or Gln that is a weak base and the residue found at the equivalent position in Ras p21 and other small GTPases, as well as in heterotrimeric G protein alpha subunits. It was found, as previously for the His-84→Gly substitution in the isolated G-domain, that the intrinsic GTPase was even for H84A, although reduced, still ~10% of the wt activity. Thus, this residue is unlikely to be the major catalytic element in the intrinsic GTPase reaction. Still, it was not possible to rule out its involvement in the ribosome-stimulated reaction, since the activity of EF-Tu[H84A] is not stimulated by ribosomes. This also prevents EF-Tu[H84A] from participating in protein synthesis. Also the prediction was tested that removing the “hydrophobic barrier” consisting of residues Ile-60 and Val-20 could increase the intrinsic GTPase activity by allowing His-84 better access to the γ -phosphate. It had already been shown that the intrinsic GTPase is reduced for mutant V20G, and in this work it is shown that mutation I60A also leads to reduced catalytic activity, rather than an increased one. Together these observations plead against a general base activation mechanism and are more in favor of another mechanism like transition state stabilization or leaving group activation.

Can mutations increase the EF-Tu GTPase?

EF-Tu has an intrinsic GTPase activity, but this catalytic action is negligible compared to the burst hydrolysis it is capable of at the moment when its complexed aa-tRNA “reads” a (near-)cognate codon in the ribosomal A site. An important question is whether EF-Tu contains all the elements necessary to express this “full” catalytic activity, for example after an induced conformation change, or whether an outside element (protein residue or RNA base) must contribute. If the first is true, one would expect that structural changes such as are induced by mutations could lead to increased (intrinsic) GTPase activity. A first indication for this was found by Harmark *et al.* in the G-domain[D80N] (Harmark *et al.*, 1992). Later preliminary characterization of full-length EF-Tu mutated in Thr-25 and purified using kirromycin suggested also an increased activity (unpublished observation). This prompted the more thorough examination of these mutations in full-length EF-Tu. They were expressed as a GST-fusion, thus avoiding the need for kirromycin to separate the mutant factor from cellular EF-Tu (Anborgh *et al.*, 1991). Surprisingly, full-length EF-Tu[D80N] displayed rather a decreased GTPase activity, while it did not have the extreme lability of the G-domain[D80N]. However, a decreased regulation of the GTPase in this mutant is indicated by the observation that in the complex with Phe-tRNA the GTPase is rather higher than for the wt (*Chapter 4*; Krab & Parmeggiani, 1999b) while the ribosome-stimulated rate is comparable. Also the mutations of position 25 that was examined did not show any sign of increased intrinsic GTPase activity, making it likely that the preliminary observations were due to some contamination with kirromycin from the purification procedure. These observations imply that even if no outside elements are contributed to the active site by the ribosome, an intricate conformational change

must be induced by codon recognition that can perhaps not be mimicked by a residue substitution in the intact EF-Tu.

Stimulation of the GTPase by kirromycin and the ribosome

Several factors, among which high monovalent salt concentrations, the antibiotic kirromycin and unprogrammed ribosomes are able to partially activate the GTPase center. For the latter two effectors the effects are additive. Evidence presented in this work suggests that these two follow different mechanism of stimulation. Firstly, the EF-Tu[T25A] GTPase is not detectably stimulated by kirromycin, whereas ribosomes can enhance its catalytic ability to an extent that is not less than for related mutants (*Chapter 3*). Secondly, kirromycin stimulates EF-Tu[T61A] GTPase comparably to that of EF-Tu[T61N], whereas ribosomes stimulate the former five times less than the latter. Known kirromycin-resistant mutations in EF-Tu mostly border the domain 1-3 interface of the GTP-bound conformation, while the influence on G-domain and EF-Tu[Δ3] (Cetin *et al.*, 1998) indicates that the main interaction of kirromycin is with domain 1. Moreover, the well-known competition between kirromycin and EF-Ts for binding to EF-Tu means that also elements from the EF-Tu/EF-Ts contact surface could be implicated, which includes the P-loop. It is possible that kirromycin exerts its GTPase stimulating action through the exceptionally strong hydrogen bond from the Asp-21 main chain NH group towards the β - γ bridging oxygen of GTP, which was proposed by Maegley *et al.* (Maegley *et al.*, 1996) to have a catalytic role in the intrinsic GTPase activity of ras p21. Interestingly, the only known mutations conferring resistance to enacyloxin IIa also cause kirromycin resistance, though the relative order of efficiency is different (Zuurmond *et al.*, 1999a). Nevertheless, enacyloxin IIa does not stimulate the GTPase activity of EF-Tu. Considering the quite different molecular structures, it is likely that the divergent effects of the two antibiotics are the consequence of specific differences in the way they bind, while sharing binding elements in the region defined by the resistance mutations.

Nucleotide binding and structural stability of EF-Tu

It has been known since long that EF-Tu of *E. coli* is not very stable and that the nucleotide plays an essential role in maintaining its functional stability. In *Chapter 6* evidence is presented that the nucleotide is not only important for maintaining the stability of EF-Tu, but also for acquiring the right conformation after EF-Tu is produced by translation in the cell. Mutants that have decreased nucleotide affinity, like EF-Tu[T25A] and the double mutant EF-Tu[H22Y/T25S] are slower in finding a correct fold, as evidenced by their decreased soluble fraction in the cytoplasm after overproduction. EF-Tu[D138N], which cannot bind any of the nucleotides available in the cell, achieves a soluble state for only a very minor fraction of the overproduced protein, approximately equal to the amount of cellular EF-Ts. The hypothesis that EF-Ts might assist in the folding of the latter mutant factor and the others that are defective in

nucleotide binding was tested by constructing a vector that combines the genes for (GST-)EF-Tu and EF-Ts in the same operon, so that they are overproduced in approximately equimolar amounts. The coexpression gave a drastic improvement of both total production and solubility for the GNP-binding-defective mutants. For the XNP-specific EF-Tu[D138N] a long delay in the appearance of soluble product was observed. Only far into the stationary phase the majority of the overproduced protein goes into solution, whereas the overproduced EF-Ts is mostly soluble from the beginning. This testifies to the importance of the nucleotide as a “folding template” while showing that EF-Ts can act as a “steric chaperon” for the folding of EF-Tu in the cell.

Nucleotide affinity of EF-Tu and stimulation of nucleotide exchange by EF-Ts

Several “dominant negative” mutations of signal transduction factor Ras p21 are known that inhibit the function of the wild type factor when present simultaneously in the cell. This has been attributed to sequestration by the mutant proteins of the nucleotide exchange factors that activate p21. It was indeed shown *in vitro* that dominant negative p21s have a reduced affinity for nucleotide and form a complex with exchange factors that is stabilized compared to wt factor, in other words, is not readily dissociated by nucleotides. When EF-Tus that are mutated in equivalent positions (Thr-25 and Asp-80) were studied, also these aspects were investigated. EF-Tu[T25A] and EF-Tu[D80N] turn out to indeed have a strongly reduced affinity for both GTP and GDP. Moreover, they interfere with the growth of cells—an inhibition that can be relieved by simultaneous overproduction of EF-Ts (*Chapter 6*)—and also inhibit the stimulation of GDP dissociation by EF-Ts on wt EF-Tu *in vitro* (*Chapters 4 and 5*). Surprisingly, however, EF-Tu[H22Y/T25S] that has a similarly decreased affinity for nucleotides, does not at all show a “dominant negative” effect. This indicates that a drastically decreased affinity for nucleotide is per se not sufficient to cause a stabilisation of the complex with EF-Ts. Since both mutations T25A and D80N affect the interaction of the magnesium ion, it is likely that the formation of the bond between EF-Tu and the nucleotide-bound magnesium is an important step on the pathway of EF-Tu•EF-Ts complex dissociation. Indeed, the phosphate binding cavity is partly closed in the EF-Tu•EF-Ts complex due to a peptide flip in the P-loop, so that a stable binding of Mg^{2+} to both these residues should be an important first step in reversing this flip. Conversely, disruption of the bond between EF-Tu and the magnesium ion is likely an important step in the stimulation of dissociation of the nucleotide by EF-Ts as proposed by Kawashima *et al.* (Kawashima *et al.*, 1996). A look at how the tertiary structure of EF-Tu might be influenced by the mutations in EF-Tu[H22Y/T25S] reveals that a steric clash between the phosphate binding loop (P-loop) and the loop L4/ α 3 region would occur, possibly forcing a reorientation of the latter α -helix. Interestingly, in the EF-Tu/EF-Ts complex crystal structure this same α -helix is displaced compared to the structure of EF-Tu•GDP. Thus this mutation might mimic a change that is on the pathway of nucleotide

dissociation stimulation by EF-Ts, increasing nucleotide release, while not hindering the important formation of a stable interaction of the Mg^{2+} -nucleotide complex with Ser-25 and Asp-80 in the first step of EF-Tu•EF-Ts complex dissociation. This would explain why it has no dominant negative properties.

Structural interpretation of properties of mutated EF-Tu

While the availability of 3D structures of EF-Tu has put into perspective a lot of known data, prediction of functional results remains difficult. On the other hand, it offers the possibility to interpret unexpected results from a structural point of view. In the course of the studies described here, two new viewpoints have arisen this way. The first concerns the interactions of the “effector region” with the rest of the molecule. From the results, removing the β -methylene group on Thr25 by mutation to Ser results in a partial destabilization of the nucleotide interaction (*Chapter 3*). This methyl group is in the wt structure surrounded by a hydrophobic “clamp” formed by two residues from the effector region: Ile49 (conserved with exceptional substitution to Val in prokaryotic/organellar EF-Tu, is Leu or Met in EF1 α) and Phe46 (invariably Phe or Tyr in prokaryotes/organelles). The latter residue is replaced by Ala in eukaryotes/archaea, but this is flanked by two aromatic residues of which the second is in most cases a Trp. This difference coincides with the substitution in these organisms of the Thr at the equivalent of position 25 by a Ser, which naturally lacks the methylene group, thus suggesting a mutual steric compensation by these substitutions.

A second novel interpretation concerns the role of residue Ile60 (*Chapter 4*). As mentioned above, the experimental results do not support the idea that this residue, as part of a “hydrophobic barrier”, serves to reduce the intrinsic GTPase of EF-Tu. An alternative answer to the question why it is so highly conserved (rarely replaced by Val and in one case by Met, from an unpublished alignment) has been proposed from the observation that in the GDP-bound conformation of EF-Tu both this residue, and the nearby Ile62 (one of the few residues universally conserved across all kingdoms) are located at the tip of the extended β -hairpin formed by unrolling α -helix 1” and form major hydrophobic contacts with domain 3 (Abel *et al.*, 1996; Polekhina *et al.*, 1996). Thus these residues help to stabilize the relative “extended” orientation of the domains, perhaps even playing a role in the conformational transition. Interestingly, the same residues are conserved also in bacterial elongation factor G and in eukaryotic EF-2, of which the structure of the effector region is not yet known, nor the high-resolution structure of the GTP-bound conformation. While the structural difference between GDP- and GTP-bound conformations of EF-G is probably much less dramatic than for EF-Tu (Czworkowski & Moore, 1997), on the ribosome the orientation of domains 3-5 changes relative to domains 1-2 (Agrawal *et al.*, 1999; Stark *et al.*, 2000), which involves an interface similar to that where the major changes in EF-Tu take place. This makes it attractive to speculate that the EF-G effector region might feature a similar helix-to loop unrolling during its

conformational changes, with the two Ile residues playing a stabilizing role by contacting one of the other domains. Higher resolution structures must answer this.

Specific action of enacyloxin IIa, a new EF-Tu antibiotic, and differences with kirromycin action

Chapter 7 describes the characterization of enacyloxin IIa, the first member of a fourth structural family (linear polyenic) of antibiotics that act on EF-Tu. It turns out to share characteristics with other EF-Tu-acting antibiotics, especially concerning the influence on nucleotide interaction. In several ways it resembles the action of kirromycin: (i) enacyloxin IIa and EF-Ts compete for binding to EF-Tu, as does kirromycin; (ii) both antibiotics still allow binding of aa-tRNA to EF-Tu, unlike pulvomycin and GE2270 A; (iii) both antibiotics induce on EF-Tu•GDP an “EF-Tu•GTP-like” conformation, allowing the GDP-bound factor to productively interact with aa-tRNA and the ribosome. On the other hand, this latter change of conformation is less strong than for kirromycin. Moreover, this new antibiotic is only transiently bound to EF-Tu, unlike the others that are very difficult, or practically impossible to remove, once bound (with the exception of kirromycin that can be removed through its competition with EF-Ts (Chinali *et al.*, 1977). Binding of aa-tRNA makes the binding of enacyloxin IIa even more labile. As a consequence of these differences, the retention of EF-Tu on the ribosome in the presence of antibiotic after aa-tRNA delivery could not be detected, in circumstances where kirromycin-induced retention does occur. Since Zuurmond *et al.* (Zuurmond *et al.*, 1999a) found that enacyloxin IIa sensitivity is dominant over resistance, like for kirromycin, this means that either enacyloxin IIa causes a slowdown of release from the ribosome that was undetectable with the techniques employed, yet is enough to cause “ribosome jam” with actively cycling ribosomes, or that the traditional model for the inhibiting action is incomplete, and that other factors play a role in inhibiting the elongation cycle with enacyloxin IIa and kirromycin.

Perspectives and conclusion

The studies described in this work have yielded a number of surprising observations that brings nearer a complete overview of the detailed mechanisms of EF-Tu function. In the characterization of a new antibiotic acting on EF-Tu, yet another tool that could be profitably employed to map the process of protein synthesis has become available. However, many questions like the nature of the triggering by the ribosome of EF-Tu’s enzymatic activity, remain difficult to answer with the techniques employed. In this regard, stop-flow techniques kinetics can discern effects on reactions too rapid to be accessible by classical experiments and measurements using fluorescent labels constitute a valuable approach to deduce the overall course of interactions as complex as those taking place on the ribosome (both employed by the group of Wintermeyer, e.g. Rodnina *et al.*, 1995; Rodnina *et al.*, 1996). Some further insight

could still be gained from additional mutations, for example of Asp80 ($\rightarrow$ Ala or Glu) and Asp50 ($\rightarrow$ Ala, Asn or Glu), that target the residues that must be involved in forming the transition state. However, so far no clear picture of the transition state could be drawn on the basis of the characterization of just such mutants. Other experiments could be suggested by the progress made concerning medium-resolution structural work on actual factor-ribosome complexes (bearing in mind that the antibiotics used to “block” complexes at a certain stage (Stark *et al.*, 1997; Agrawal *et al.*, 1999; Stark *et al.*, 2000) could create a situation not representative of the normal interaction). While now both Thr61 and His84 are known to be in some way important for the ribosomal stimulation of hydrolysis, other residues that are not adjacent to the catalytic center but are known to be defective in ribosome interaction, like B₀ (G222D) and G280V (Tubulekas & Hughes, 1993b), can also inhibit ribosome triggering (Swart *et al.*, 1987; Vorstenbosch *et al.*, 1996). Moreover, the direct interaction of ribosomal RNA or RNA mimics (Hornung *et al.*, 1998) with EF-Tu deserves to be further examined for its functional implications. Also the involvement of the aa-tRNA which is necessary for generating the trigger signal needs further clarification (Piepenburg *et al.*, 2000), as well as the pathway of the conformational transitions both in GTP hydrolysis and in EF-Ts complex formation and dissociation. Such dynamic changes of a protein that goes through a complex cycle with many different interactions remain a challenge for our ability to creatively exploit both structural and functional data in drawing a concrete picture of its functioning.

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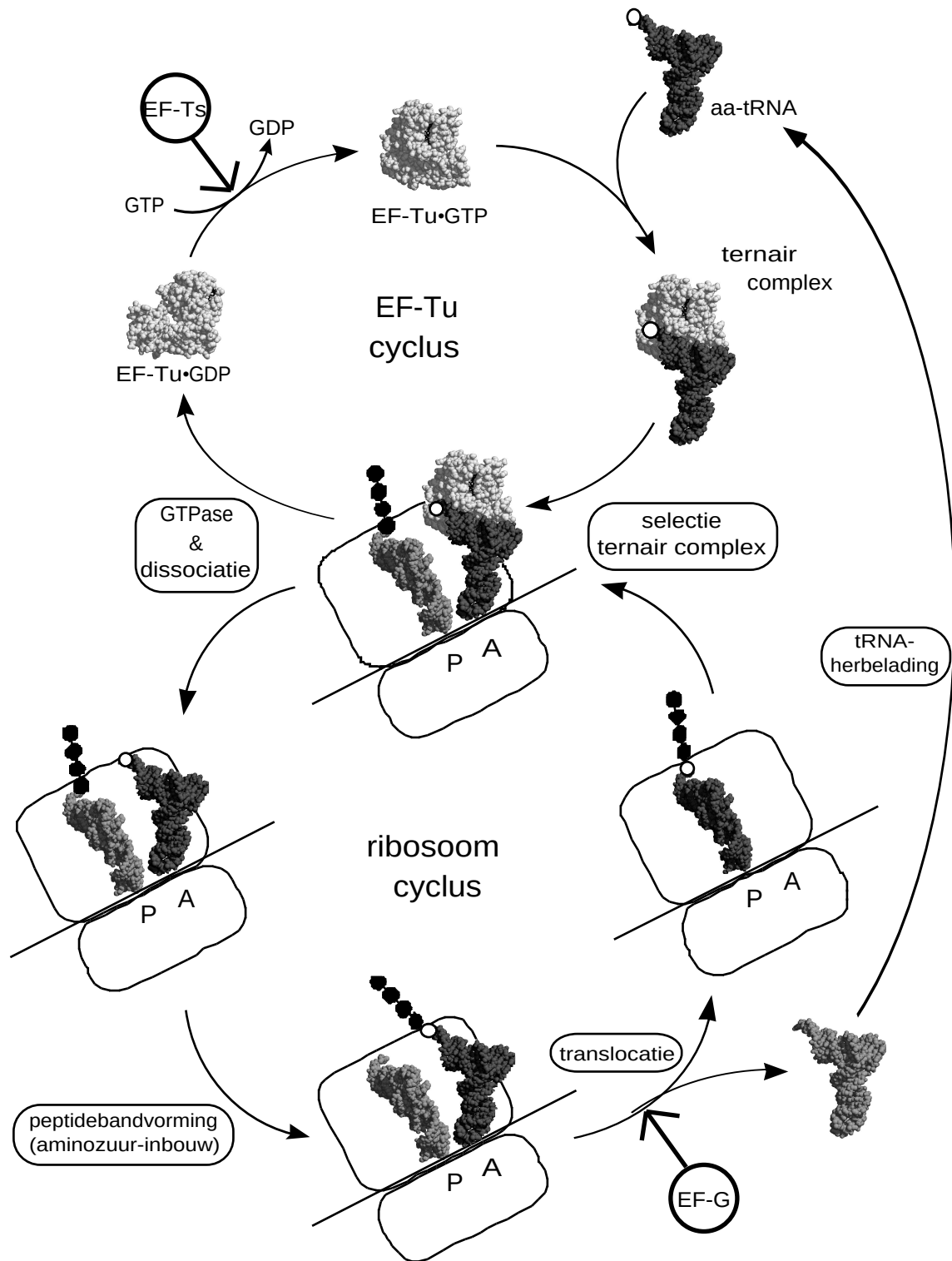
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Figuur 1. Schematisch overzicht van de elongatiecyclus. Zie de tekst voor een beschrijving. EF-Tu en het (aa)-tRNA zijn weergegeven als atoommodellen van de experimenteel bepaalde structuren. Het nieuw in te bouwen aminozuur is voorgesteld als een witte “kraal”, en de eerder ingebouwde aminozuren van de groeiende keten als een ketting van (vier) zwarte kralen. Het ribosoom is heel schetsmatig aangeduid, met een grote en een kleine “box” voor de grote en de kleine subeenheden, en een streep in het midden voor het boodschapper-RNA waaraan de tRNA’s binden. Het punt waar EF-Ts en EF-G tussenbeide komen in de cycli is aangeduid met pijlen.

Samenvatting

Eiwitten zijn grote moleculen met bijzondere eigenschappen waardoor ze een universeel gereedschap vormen voor levende cellen¹. Cellen besteden dan ook een groot deel van hun beschikbare middelen aan de productie van eiwitten, die zulke diverse rollen spelen als: 1) enzymen, die andere moleculen kunnen omvormen om benodigde stoffen te maken; 2) transporteurs, die moleculen op de gewenste plaats in de cel afleveren; 3) structurele elementen, die vorm en stevigheid geven aan de cel; 4) motorelementen, die voor beweeglijkheid zorgen; 5) opslag van bouw materiaal, denk aan het eiwit in een ei.

Eiwitten worden gesynthetiseerd in een tweestaps-proces. De assemblage-instructies, opgeslagen in het DNA-molecule in eenheden die *genen* heten, worden eerst getrouw gekopieerd in een ‘boodschapper-RNA’-molecule (engelse afkorting: *mRNA*). Deze stap heet *transcriptie*. Vervolgens worden deze gekopieerde instructies gelezen en ‘vertaald naar’ eiwit door een moleculaire machine, het *ribosoom*. Dit proces noemen we *translatie*. Tijdens de translatie worden aminozuren stap voor stap aaneengeregen door het ribosoom in een cyclisch proces, de *elongatiecyclus* (zie Figuur 1 hiernaast). De benodigde aminozuren worden aangeleverd in een chemisch geactiveerde vorm, gekoppeld aan een adaptermolecule dat *tRNA* heet. Er zijn specifieke tRNA’s voor elk aminozuurtype, en ze hebben ieder een herkenningsstructuur die als een puzzelstuk past op de instructie voor de inbouw van ‘hun’ aminozuur in het boodschapper RNA-molecule. Zo is de vertaling van instructies naar eiwit in principe niet anders dan de selectie op het ribosoom van steeds het passende tRNA-gekoppelde aminozuur (afkorting: *aa-tRNA*) uit het veertigtal beschikbare. Deze stap wordt aanzienlijk versneld en nauwkeuriger gemaakt door een hulpeiwit dat *elongatiefactor Tu* (afkorting *EF-Tu*) heet, een transporteiwit voor aa-tRNA’s.

Wanneer EF-Tu het juiste benodigde tRNA-gebonden aminozuur aflevert op het ribosoom, induceert de puzzelstuk-interactie van dit aa-tRNA met het boodschapper RNA een enzymatische activiteit van EF-Tu. Daardoor modificeert het een klein molecule dat ook gebonden is aan het complex, een *nucleotide* (GTP, dat wordt omgezet in GDP). Deze kleine modificatie heeft een grote structuurverandering van EF-Tu tot gevolg, waardoor het in een inactieve toestand komt en loslaat van het aa-tRNA en het ribosoom. Het ribosoom koppelt nu het afgeleverde aminozuur aan de groeiende keten, en een ander hulpeiwit, EF-G schuift het boodschapper RNA één instructie op door het ribosoom, zodat een nieuwe cyclus kan beginnen. Het EF-Tu wordt weer geregenereerd naar een actieve toestand dat een volgend aa-

¹ een verdere uitleg over wat eiwitten zijn en over een aantal hier gebruikte termen is apart bijgevoegd (blad “Achtergrondinformatie voor niet-specialisten”)

tRNA kan binden door een hulpeiwit, EF-Ts, dat de uitwisseling van het gebonden GDP-nucleotide voor een nieuw GTP stimuleert. Figuur 1 geeft een schematisch overzicht van deze cyclus.

Dit proefschrift beschrijft studies van het EF-Tu van de darmbacterie *Escherichia coli*, het standaard “werkpaard” van de moleculair-biologische wetenschap. Het opent met een discussie van de staat van kennis over EF-Tu en het proces van eiwitsynthese, dat op moleculair niveau bestudeerd is sinds zuivere componenten van het systeem voor het eerst geïsoleerd konden worden in de jaren zestig van de vorige eeuw. Hoofdstuk 2a (gepubliceerd als een overzichtsartikel in 1998) geeft een overzicht van de bevindingen van bijna dertig jaar onderzoek aan EF-Tu en relevante andere elementen van de eiwitbiosynthese-machinerie zoals gepubliceerd in de wetenschappelijke literatuur. Hoofdstuk 2b voegt daaraan enkele recenter ontwikkelingen toe.

De belangrijkste vraagstelling van dit proefschrift was te achterhalen wat de precieze rol is van een aantal aminozuurresiduen die geconserveerd zijn rondom de plaats waar het GDP- of GTP-nucleotide gebonden is aan het EF-Tu. Het betreft threonine-25, isoleucine-60, threonine-61, aspartaat-80 en histidine-84. Twee van deze —Thr-25, Thr-61— binden direct, en Asp-80 indirect, aan een magnesium-ion dat weer aan de fosfaatgroepen van het nucleotide gebonden is. Er waren aanwijzingen dat deze residuen belangrijk zijn voor de enzymatische omzetting van het nucleotide door EF-Tu. Bijvoorbeeld, mutatie van Asp-80 naar asparagine in een ingekort EF-Tu-molecule dat alleen het enzymatisch domein omvat, versnelde de intrinsieke (niet-gestimuleerde) reactie enkele tientallen malen, maar destabiliseerde ook het EF-Tu-domein aanzienlijk. Ook een mutatie van Thr-25 leek in een eerste onderzoek de GTPase¹ te versnellen. Verder was er een theorie, dat His-84 een directe katalytische rol speelt, waarbij Ile-60 samen met valine-20 een barrière zou vormen, die de intrinsieke katalytische activiteit laag houdt, zodat geen energie onnodig verloren gaat.

Hoofdstukken 3-5 nemen de invloed op de katalyse en andere meetbare karakteristieken van EF-Tu van mutanten van al deze aminozuren onder de loep. Wat de katalyse betreft, zijn de bevindingen in tegenspraak met zowel de hypothese dat His-84 een katalytische rol speelt in de intrinsieke GTPase, als met het idee dat Ile-60 een barrière-functie vervult. Vreemd genoeg vertoont de mutant Asp-80→asparagine in het hele EF-Tu geen spoor van de verhoogde katalytische activiteit die het in een ingekort molecule had, noch is de stabiliteit drastisch verminderd. Er is echter wel een punt dat wijst op een invloed op de normaal-gesproken hechte regulatie van de GTPase met deze variant: de binding van aa-tRNA, die normaal de GTP hydrolyse nog verder vermindert heeft hier een aanzienlijk stimulerend effect. Ribosomen en het antibioticum kirromycine kunnen beide de GTPase van EF-Tu stimuleren. Verschillen die in dit onderzoek naar boven kwamen in de activatie van mutanten van Thr-25 en Thr-61 door deze

¹ GTPase is de term voor het type reactie dat EF-Tu katalyseert. Ook wel hydrolyse van GTP genoemd

twee geven een duidelijke indicatie dat hun stimuleringsmechanisme verschilt.

De centrale rol die het magnesium-ion speelt in de binding van het nucleotide wordt bevestigd door een voorspelbare drastische vermindering van de GDP- en GTP-bindingsaffiniteit door de mutatie Thr-25→Ala, en minder voorspelbaar, door de substitutie Asp-80→Asn. Een mutant met twee veranderde aminozuren, His-22→Tyr en Thr-25→Ser, heeft echter geheel onverwachts een eveneens sterk verminderde affiniteit voor nucleotiden. Deze dubbelmutant vormt daarentegen geen ongewoon stabiel complex met EF-Ts, wat Thr-25→Ala en Asp-80→Asn wél doen. Daaruit kan worden afgeleid dat voor stabilisatie van het complex EF-Tu•EF-Ts niet een verlaagde nucleotide-affiniteit, maar een veranderde interactie met het magnesium ion verantwoordelijk is. Dit geeft ook aan, dat in het mechanisme van nucleotide-dissociatie door EF-Ts het losmaken van het magnesium-ion een belangrijke rol speelt, zoals eerder was gesuggereerd op basis van de structuur van het EF-Tu•EF-Ts complex.

In een poging om de experimentele bevindingen te rationaliseren is ook naar de bekende ruimtelijke structuren van EF-Tu gekeken. Daaruit is een tweetal nieuwe inzichten voortgekomen. Het eerste betreft de reden dat er in EF-Tu op positie 25 een threonine voorkomt, terwijl andere eiwitten met een soortgelijk GTPase-domein hier vaak een serine bezitten. Een belangrijk deel van het katalytisch domein, dat het “effectorgebied” heet, blijkt namelijk de zijketen van Thr-25 “in de tang” te nemen, en wordt zo gestabiliseerd (Figuur 6 A&B op p. 51). Het tweede inzicht betreft de reden dat Ile-60 zo sterk geconserveerd is, terwijl de eerder voorgestelde rol als “barrière” die het energieverlies laag houdt niet blijkt te kloppen. Het blijkt namelijk dat het centraal ligt in een gebied dat drastisch van structuur verandert bij de overgang van de GTP-gebonden naar de GDP-gebonden vorm van EF-Tu. In de GDP-gebonden vorm vormt dit gebied een “brug” van het katalytisch domein naar domein 3. Deze domeinen staan veel losser met elkaar in contact dan in de GTP-gebonden vorm, en isoleucine-61, samen met isoleucine-63, dat óók sterk geconserveerd is, vormt een belangrijk contactpunt van de “brug” met domein 3 (zie Figuur 5 C op p. 65). Op basis van dit inzicht kunnen we speculeren, dat ook in nauw aan EF-Tu verwante eiwitten als EF-G en IF-2, waar dezelfde residuen geconserveerd zijn, maar waarvan de structuur van het effectorgebied niet bekend is, een dergelijke structuurverandering zou kunnen plaatsvinden.

Een tweede vraag die tijdens het onderzoek opkwam, betreft de relatie tussen de affiniteit voor guanosine-nucleotiden en de stabiliteit/vouwbaarheid van EF-Tu-varianten. Een verminderde affiniteit bleek namelijk te correleren met een verminderde oplosbaarheid van het eiwit bij translatie in de cel, wat aangeeft dat het eiwit niet correct opvouwt. In het meest extreme geval, dat van de mutant Asp-138→Asn was de hoeveelheid oplosbaar eiwit nooit hoger dan de hoeveelheid EF-Ts in de cel, wat suggereerde dat EF-Ts een rol kon hebben in de vouwing van het EF-Tu. Het is sinds lang bekend dat EF-Tu van *E. coli* in afwezigheid van guanine-nucleotiden onstabiel is, maar dat het nucleotide-vrije complex EF-Tu•EF-Ts een veel hogere

stabiliteit vertoont. Deze kwestie werd onderzocht door een speciale expressievector te maken die EF-Tu en EF-Ts in gelijke hoeveelheden kan overproduceren, doordat beide genen als één cistron overgeschreven worden. Verscheidene EF-Tu-mutanten die een verminderde nucleotide-affiniteit hebben werden in deze vector geproduceerd, en in alle gevallen werd een aanzienlijke toename in de hoeveelheid oplosbaar produkt verkregen. Interessant is, dat in het geval van EF-Tu Asp-138→Asn het produkt in eerste instantie niet oplosbaar is, maar na verlengde incubatie alsnog hervouwt en in oplossing gaat, zelfs als verdere eiwitproductie geblokkeerd is. Dit geeft aan, dat het nucleotide normaal een belangrijke rol speelt in de vouwing van EF-Tu in de cel, maar dat EF-Ts deze rol over kan nemen, en zo als een “sterische chaperone” kan fungeren (zie Hoofdstuk 6).

Het laatste onderzoeksthema in dit proefschrift betreft de karakterisering van het werkingsmechanisme van een nieuw antibioticum, enacyloxine IIa, dat EF-Tu als aangrijpingspunt heeft (zie Hoofdstuk 7). Enacyloxine IIa vertegenwoordigt een vierde chemisch onderscheiden klasse van EF-Tu-specifieke antibiotica, naast de drie typen die al bekend waren. Het deelt een aantal karakteristieken met de andere drie, maar lijkt het meest op kirromycine, in zoverre dat het a) wedijvert met EF-Ts voor binding aan EF-Tu, b) als kirromycine —maar in tegenstelling tot pulvomycine en GE2270 A— binding van aa-tRNA aan het EF-Tu•antibioticum complex toestaat, c) net als kirromycine in EF-Tu•GDP een “EF-Tu•GTP-achtige” structuur induceert, waardoor het EF-Tu•GDP•antibioticum complex produktief met aa-tRNA en het ribosoom kan wisselwerken. Deze structuurverandering is echter minder uitgesproken dan in het geval van kirromycine. Een opvallend verschil met de andere antibiotica is de lage stabiliteit van de binding van enacyloxine IIa aan EF-Tu. Terwijl de andere antibiotica wanneer ze eenmaal gebonden zijn niet meer van EF-Tu te scheiden zijn (alleen kirromycine kan door gebruik te maken van de competitie met EF-Ts verwijderd worden), kan enacyloxine IIa heel gemakkelijk van EF-Tu gescheiden worden. Binding van aa-tRNA verzwakt de interactie nog meer. Het was dan ook niet mogelijk om vast te stellen of EF-Tu•GDP•enacyloxine IIa op het ribosoom achtergehouden wordt na enzymatische binding, zoals het geval is met kirromycine. Kirromycine kan in heel lage hoeveelheden al de eiwitsynthese platleggen, en gevoeligheid is dominant over resistentie, wat betekent dat als er een mengsel van gevoelig en resistent EF-Tu aanwezig is, eiwitsynthese toch geremd wordt. Het gebruikelijke mechanisme dat hiervoor ingeroepen wordt, is de “verkeersopstopping” op mRNA, waar één ribosoom dat geblokkeerd is door EF-Tu•kirromycine ook navolgende ribosomen ophoudt. Dit effect werd door Zuurmond e.a. (1999a) ook voor enacyloxine IIa gevonden. Omdat enacyloxine echter EF-Tu niet sterk op het ribosoom vasthoudt, betekent dit dat slechts een relatief kleine vertraging van EF-Tu-dissociatie van het ribosoom voldoende zou zijn om een “verkeersopstopping” te veroorzaken, òf dat het blokkeringsmechanisme toch iets gecompliceerder is dan voorheen voorgesteld.

Curriculum Vitae

Ivo Maarten Krab

Geboren 31 januari 1970 te Capelle a/d IJssel

apr. 1988	Eindexamen VWO aan de Christelijke Scholengemeenschap Comenius te Capelle a/d IJssel
sep. 1988-apr. 1993	Studie scheikunde aan de Universiteit Leiden, met specialisatie in de Biochemie (Dr. C.W.A. Pleij)
apr. 1993-sep. 1999	Promotie-onderzoek aan de Ecole Polytechnique, Palaiseau, Frankrijk onder supervisie van Dr. A. Parmeggiani. Promotor: Prof. Dr. C.W.A. Pleij, Universiteit Leiden
dec.1999-jun. 2000	Korte onderzoeksstage aan de Universiteit Leiden in de groep van Dr. B. Kraal
Vanaf aug. 2000	Centre for Protein Engineering, Universiteit Cambridge, onderzoek aan de simulatie van eiwitvouwing in de groep van Prof. Dr. A. Fersht

Tijdens het promotie-onderzoek werd deelgenomen aan de jaarlijkse bijeenkomsten van het “Human Capital and Mobility Network on Molecular Mechanisms of EF-Tu” (Oegstgeest 1995, Ischia (Italië) 1996, Spetsai (Griekenland) 1997 and Mülltal (Duitsland) 1998). Daarnaast werd deelgenomen aan het internationale congres “Frontiers in Translation, an International Conference on the Structure and Function of the Ribosome” (20-25 mei 1995) in Victoria, Canada.

Nawoord

This thesis represents the endpoint of a long voyage, both literally and in terms of personal development. It was quite a change to leave home not to go to a student room in the next city, with weekends for bringing the laundry home to mum as many would do, but to another country altogether. The first three years I lived in the house of the Soulier family (M. et Mme., je suis très reconnaissant de votre accueil généreux et chaleureux). Also at the lab I was received very well into the group. Andrea, with untameable energy, affronted the many obstacles that littered the administrative course our always fragile group was sailing, and largely contributed to shape my ideas of what being a scientist involves. Other colleagues, several of who became close friends, taught me valuable skills and provided interesting discussions, practical help and moral support. Of these, I would especially like to mention Rengül, who was in the not very enviable situation of having her husband far away for over a year, and then having a baby while trying to finish her thesis, Carmela, always good for some nice fireworks with her southern temperament but with a kind heart underneath, Devrim, who unfortunately stayed only a while, then disappeared out of sight, and Rob, who chose to return to our lab to see the last two years of its existence. I also enjoyed the company of the others that sailed the rocky ship variously called SDI n° 61840 and BIOP Equipe 2: co-PhD students Maria-Carla, unperturbable and determined, Soria, lively and ‘toujours de bonne humeur’, Ab, the ‘bon vivant’ and Piet, already a Doctor when I arrived and leaving shortly after to ‘cross the pond’, and the ‘crew’ consisting of Jean-Bernard (who introduced me to ‘la muscu’; although I’m afraid I just didn’t have the discipline to continue) and Eric, both supportive and always ready for a chat. For a short period Giuliano Scarano came to Palaiseau as an exchange with the Naples lab of Prof. Bocchini and was assigned as ‘my’ student. The majority of the experiments resulting in Chapter 5 were carried out by her. I was sorry to see her go, and to hear she shortly after left the research field for personal reasons. All of you made the Palaiseau time unforgettable.

While the French Connection covers most of this episode, I shouldn’t forget the tie to my ‘homebase’ Leiden. The research I did could result in this thesis thanks to the kind agreement of Kees Pleij to continue the long-standing tradition of Dutch PhD students being delegated to Palaiseau, while being ‘Doctored’ in Leiden. It sadly will not happen again, now. Fortunately Barend Kraal and Kees Pleij allowed me to tie up the last strings of my thesis in Leiden, after the official and irrevocable demise of the Palaiseau lab. It was enjoyable to find old acquaintances back there: particularly Gilles, Anne-Marie and Gert-Jan provided the same incomparable “HB129” atmosphere that I had left behind years before.

And through it all, I received the unconditional support of my parents and my brother, which was very precious to me in this decisive phase of my life. Finally, I say to the one who comes first place: Pascal, words are not enough....