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Isolation and functional analysis of histidine-tagged elongation factor Tu

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The study of the structure/function relationships of the *Escherichia coli* elongation factor Tu (EF-Tu) via mutagenesis has been hampered by difficulties encountered in separating the mutated factor from other proteins, in particular native EF-Tu. Here we describe a novel system for the purification of EF-Tu mutant species, based on metal-ion affinity chromatography. To facilitate rapid and efficient purification we designed a recombinant EF-Tu with an additional C-terminal sequence of one serine and six histidine residues. A cell extract containing the His-tagged EF-Tu (EF-TuHis) is applied to a Ni^{2+}-nitrilotriacetic acid column. EF-TuHis can be selectively eluted with an imidazole containing buffer, yielding a preparation of more than 95% purity, free of wild-type EF-Tu. *In-vitro* and *in-vivo* functional analyses show that EF-TuHis resembles the wild-type EF-Tu, which makes this one-step isolation procedure a promising tool for the study of the interactions of mutant EF-Tu with the various components of the elongation cycle. The new isolation procedure was succesfully applied for the purification of a mutant EF-TuHis with a Glu substitution for Lys237, a residue possibly involved in the binding of aminoacyl-tRNA.

The elongation factor Tu (EF-Tu) plays an essential role in the elongation cycle of prokaryotic protein synthesis. In complex with GTP, EF-Tu binds Xaa-tRNA forming a ternary complex that can bind to the vacant A-site on the translating ribosome. After GTP hydrolysis the EF-Tu·GDP leaves the ribosome. The recycling of EF-Tu·GDP is stimulated by the elongation factor Ts [1].

Many biochemical studies have been carried out to elucidate the mechanism by which EF-Tu·GTP interacts with Xaa-tRNA. It is known that EF-Tu·GTP primarily recognizes three regions of Xaa-tRNA. These include the aminoacyl group, the CCA 3′-terminal sequence and the adjacent acceptor arm (for review see [2]). Much less is known about the sites of EF-Tu that are involved in the binding of Xaa-tRNA. Numerous studies using chemical modification [3–6], limited trypsin cleavage [7–8] and cross-linking [9–13], show that Xaa-tRNA has many contact regions in EF-Tu, mostly located in the N-terminal and middle domain of EF-Tu. *In-vitro* site-directed mutagenesis may further extend our understanding of this interaction. However, a complicating factor in the purification of mutant EF-Tu proteins is the presence of large amounts (5–10% of the total cellular proteins) of the chromosomally encoded EF-Tu. Usually the change of one or two amino acids does not lead to a major change in the physical properties of the protein, which makes it difficult to completely separate the mutant EF-Tu from the wild-type EF-Tu. The latter is essential for the *in-vitro* study of the effect of the mutation on the interaction between EF-Tu and Xaa-tRNA. Traces of wild-type EF-Tu would interfere in the interaction, thus masking the effect of the mutated amino acid in the protein.

A variety of genetic approaches has been used to facilitate protein purification. Examples include fusion of the coding sequence to protein A or β-galactosidase, but often these fusion proteins are large and may be biologically inactive [14]. A few years ago a procedure was reported for the purification of EF-Tu mutant proteins based on the differences in affinity for the antibiotic kirromycin and the competition of elongation factor Ts and kirromycin for the binding to EF-Tu [15–17]. Although this method has been successfully used for the isolation of EF-Tu mutants, the procedure is dependent on the affinity of the EF-Tu mutant for the antibiotic, implying that mutants affected in the binding of kirromycin cannot be isolated. We therefore decided to develop an alternative purification procedure based on metal-ion affinity chromatography. Proteins containing a sequence of neighbouring histidine residues can be purified using a Ni^{2+}-charged nitrilotriacetic-acid affinity column in a one-step procedure. In this way various proteins have been purified, all containing a stretch of six histidine residues at the N-terminus or C-terminus of the protein [18–22].

Here we report the application of this approach to the purification of mutant EF-Tu proteins. To this aim we engineered a recombinant EF-Tu with an additional C-terminal tag consisting of one serine and six histidine residues and developed an improved elution procedure for the affinity purification. We demonstrate that the biological activity *in vitro* and *in vivo* of the His-tagged EF-Tu (EF-TuHis) is comparable to that of the wild-type EF-Tu, making the method suitable for the functional analysis of mutant EF-Tu species in general. This method was used for the isolation of the

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Abbreviations. Xaa-tRNA, aminoacyl-tRNA; EF-Tu, *E. coli* elongation factor Tu; EF-Tu His, EF-Tu with additional C-terminal tag of Ser-His_6.

Enzyme. Pyruvate kinase (EC 2.7.1.40)

mutant [K237E]EF-TuHis. The substitution Lys237→Glu was performed to study the involvement of Lys237 in the binding of Xaa-tRNA.

MATERIALS AND METHODS

Construction of pKECAHis

All DNA manipulations were performed following standard protocols given by Sambrook *et al.* [23]. Site-directed mutagenesis was performed according to Kunkel et al. [24], using M13mp18. The *tufB* gene was isolated from the plasmid pTuB11.2 [25] as a 1.7-kbp *Pst*I – *Bam*HI fragment and ligated into the *Pst*I/*Bam*HI sites of the vector M13mp18. The *Sac*I site of the polylinker was destroyed by digestion with *Sac*I, followed by filling of the 3′ protruding ends with T4 DNA polymerase. Using *in-vitro* site-directed mutagenesis, the TAA stop codon was mutated into a TCA codon thereby creating a unique *Sac*I site. A 22-bp synthetic oligonucleotide was ligated into the *Sac*I/*Hin*dIII sites of the M13mp18tufB, introducing a sequence of one serine and six histidine codons at the 3′ end of the gene followed by a stop codon. Finally the codon AGC, encoding serine 393, was changed into GGC, coding for a glycine residue (Fig. 1). pKECAHis was constructed by inserting the engineered gene as a 1.2-kbp *Bam*HI – *Hin*dIII fragment into the *Sma*I/*Hin*dIII sites of the polylinker of the expression vector pKK223-3 (Pharmacia). In this way the gene was placed under the control of the strong and inducible *tac* promoter.

Site-directed mutagenesis

The mutated gene was obtained by mutagenesis according to Kunkel et al. [24], using M13mp18 containing the gene encoding EF-TuHis. 5′-CCAACTTCGATGATA-3′ was used as the mutagenic primer for the mutation Lys237→Glu. The 1.2-kbp *Eco*RI – *Hin*dIII fragment containing the mutated gene was cloned into the *Eco*RI/*Hin*dIII sites of pKK223-3.

Overproduction of EF-TuHis or [K237E]EF-TuHis

Escherichia coli strains used for the expression of pKECAHis or pKECHisLys237 were JM109 [23], KA797 [*F′prolac (prolac) ara thi su*$^-$] (strain collection, Department of Molecular Genetics, Leiden University) and PM1455 [*tufA, tufB::(Mu)rpoB, recA56*] [26]. Expression of EF-TuHis and the mutated factor was induced by addition of 1 mM isopropyl-thio-β-D-galactoside to cells grown in Luria-Bertani medium at 37°C, to A_{660} 0.25, followed by an additional incubation of 3 h.

Purification of EF-Tu and EF-TuHis

Wild-type EF-Tu·GDP (*E. coli* strain MRE600) was prepared as described [27]. The nitriloacetic-acid resin was synthesised and charged with Ni^{2+} according to Hochuli et al. [18]. The prepared Ni^{2+}-nitriloacetic-acid resin was identical to the Ni^{2+}-nitriloacetic-acid agarose (Qiagen) that was used in a later stage of this study.

After induction, the cells were collected by centrifugation ($5000 \times g$, 10 min), washed and resuspended in buffer A (50 mM Tris/HCl, pH 7.6, 60 mM NH_4Cl, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 15% (by vol.) glycerol). All further steps were performed at 4°C. The cells were disrupted by sonication and a cell extract was prepared by two successive centrifugation steps at $30000 \times g$ for 30 min (S30) followed by centrifugation at $100000 \times g$ for 2 h (S100).

Proteins were eluted from the Ni^{2+}-nitriloacetic-acid column according to three different methods. In method 1 a pH gradient from pH 8.0 to 5.8 (100 mM NaH_2PO_4/Na_2HPO_4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μM GDP) was applied as described by Gentz et al. [20]. In method 2 an imidazole gradient from 0.8 mM to 80 mM in buffer A, 50 mM NaCl, 5 μM GDP was used. In method 3 elution was performed with a pH gradient from pH 8.0 to 7.0 in buffer A, 300 mM KCl, 5 μM GDP followed by elution with 80 mM imidazole in buffer A at pH 7.0, 5 μM GDP.

The fractions containing the purified protein were pooled and concentrated using Amicon Centriflo ultrafiltration cones. Protein concentration was determined by the method of Bradford [28] using bovine serum albumin as a standard.

Purification of [K237E]EF-TuHis

[K237E]EF-TuHis was prepared according to method 3 as described for EF-TuHis.

Isoelectric focusing

Isoelectric focusing was performed according to [29]. A pH gradient from pH 5 to 7 was used. The ampholytes were purchased from LKB Pharmacia.

Guanine-nucleotide-binding activity

GDP-binding activity was determined by nitrocellulose filtration. Reaction mixtures (100 μl) contained 5 μM EF-Tu or EF-TuHis, 95 μM [^{3}H]GDP (specific activity 498 cpm/pmol), and 50 μg bovine serum albumin in 64 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol. After incubation at 30°C for 10 min, the reaction was stopped and rapidly filtered through 0.45 μm nitrocellulose filters according to [30]. The amount of retained [^{3}H]GDP on the filter was determined by scintillation counting.

GTPase activity

The GTPase activities were measured by incubation with [γ-^{32}P]GTP at 30°C according to [31]. The EF-Tu·[γ-^{32}P]GTP complex was formed by incubation of 5.5 μM EF-Tu · GDP with 35.5 μM [γ-^{32}P]GTP (specific activity 3125 cpm/pmol), 2 mM phospho*enol*pyruvate and 50 μg/ml pyruvate kinase at 30°C for 5 min. The reaction was started by the addition of KCl to 1 M final concentration, with or without 50 μM kirromycin. Aliquots were taken and diluted with 1/4 volume of 25% (by vol.) formic acid at 0°C, whereafter the samples were spotted onto a cellulose thin-layer plate (J. T. Baker Inc.) that was eluted with 0.5 M formic acid and 0.5 M LiCl. The liberated $^{32}P_i$ and the [γ-^{32}P]GTP spots were scanned with a Betascope 603 (Betagen).

Electrophoresis under native conditions

Native gel electrophoresis was performed as described [32]. 5 μM EF-Tu·GDP or EF-TuHis·GDP was incubated at 37°C for 10 min with 500 μM GTP, 500 μM phospho*enol*pyruvate, 100 μg/ml pyruvate kinase and 100 μM Xaa-tRNA or 100 μM kirromycin in electrophoresis buffer (10 mM Tris/borate,

pH 7.6, and 5 mM $MgCl_2$). Immediately prior to electrophoresis 4% (by vol.) of dimethyl sulfoxide was added to the samples. The aminoacylation of bulk tRNA was performed according to [33].

In-vitro translation assay

An *in-vitro* prepared run-off transcript from the plasmid pT7.1tufB was used in the translation assay (unpublished results). For this assay an EF-Tu-free S30 extract was incubated with 18 nM mRNA in translation buffer (50 mM Tris/acetate, pH 7.6, 9 mM magnesium acetate, 75 mM potassium acetate, 1 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 6 mM phospho*enol*pyruvate, 10 μg/ml pyruvate kinase, 8% (by vol.) glycerol, leucovorine (60 μg/ml), 2% (by vol.) mixture of ^{14}C-labelled amino acids (50 μCi/ml, 56 mCi/mg.at. C, Amersham) supplemented with 0.04 mM lacking amino acids, at 37 °C for 30 min. The total volume was 100 μl. The reaction was stopped by addition of 120 μl 0.1 M NaOH and further incubation at 37 °C for 5 min. After precipitation with 7% (mass/vol.) trichloroacetic acid the amount of incorporated ^{14}C-labelled amino acids was determined.

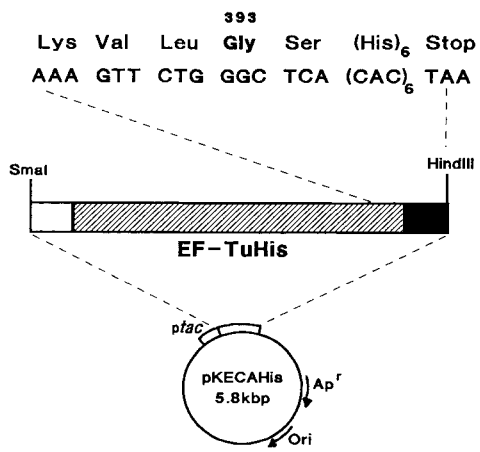

Fig. 1. The gene encoding EF-TuHis. The engineered gene consists of the coding sequence for EF-Tu, extended with one serine and six histidine codons immediately followed by a stop codon. The gene was cloned in the *Sma*I/*Hin*dIII sites of the expression vector pKK223-3, thereby placing the gene under the control of the inducible *tac* promoter. Ap^r, ampicillin resistance; Ori, origin of replication.

RESULTS

Construction and expression of the gene encoding the EF-TuHis

In the previously described method [20] a stretch of histidine residues is attached to the N-terminus of the protein of interest. The variable C-termini of EF-TuA and EF-TuB and the location of the C-terminus close to the periphery of the crystal structure [34], suggested to us that the best chance of avoiding loss of biological activity was to add a stretch of six histidine residues to the C-terminus of EF-Tu rather than to its N-terminus. Since a glycine residue, in contrast to other residues, is very slowly removed by carboxypeptidase A [19], we decided to replace Ser393 by Gly, such as present in EF-TuA. This created the possibility of removing the histidine tag, in case the modified EF-Tu proved to be inactive. The resulting engineered gene encodes EF-TuHis, the EF-Tu sequence ending with Gly393 followed by an additional seven amino acids, consisting of one serine and a sequence of six histidine residues as is depicted in Fig. 1.

The construct pKECAHis was transformed into different *E. coli* strains. Expression of EF-TuHis was induced by addition of 1 mM isopropyl-thio-β-D-galactoside. Only a minor growth inhibition could be observed following this addition. Total protein samples were prepared from cells before and after induction to determine the level of expression by SDS/PAGE and Western blotting. The results showed that it was possible to induce pKECAHis in *E. coli* strain KA797 to a high level of expression without significant growth problems and that the level of expression became maximal after 3 h of induction. The overexpressed protein migrates during SDS/PAGE like wild-type EF-Tu and reacts with the polyclonal antibodies raised against wild-type EF-Tu (Fig. 2A and B).

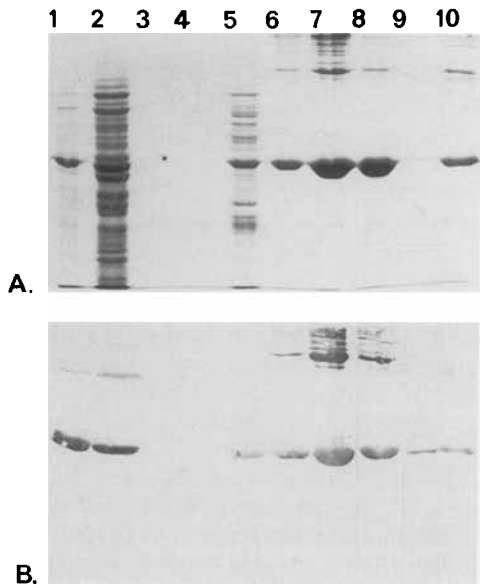

Fig. 2. Purification of His-tagged EF-Tu. Proteins were eluted according to method 3 described in Materials and Methods. (A) Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Lane 1, S100 extract applied to the column; lane 2, flow-through pH 8.0; lanes 3 and 4, wash fractions pH 7.0; lanes 5–9, elution with 80 mM imidazole; lane 10, EF-TuHis purified according to method 1. (B) Corresponding Western blot using antibodies raised against wild-type EF-Tu.

Purification of EF-TuHis

After disruption of the cells, an S100 extract was prepared and applied to the Ni^{2+}-nitriloacetic-acid column (see Materials and Methods). Three different elution methods were tested.

In the first method, a pH gradient from pH 8.0 to 5.8 was used as described by Gentz et al. [20]. Analysis of the fractions by SDS/PAGE and Western blotting showed that the majority of the proteins were found in the flow-through of the column. At pH 6.6 a protein started to elute that migrated with the wild-type EF-Tu and cross-reacted with the antibodies raised against the wild-type EF-Tu. The collected fractions were concentrated by ultrafiltration and stored in buffer A containing 15% (by vol.) glycerol and 20 μM GDP at −80 °C. The purity of the protein was estimated to be higher than 95% as determined by Coomassie Brilliant Blue and silver staining

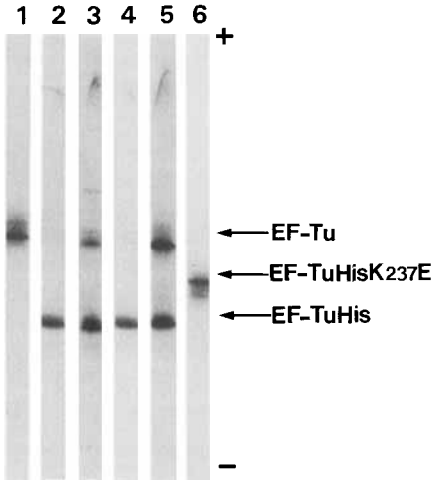

Fig. 3. Isoelectric focusing of purified EF-TuHis and [K237E]EF-TuHis. A pH gradient from pH 5 to 7, indicated (+) and (−) respectively, was used. Protein bands were visualized by staining with Coomassie Brilliant Blue. Lane 1, wild-type EF-Tu; lane 2, EF-TuHis purified according to method 1; lane 3, EF-TuHis purified according to method 2; lane 4, EF-TuHis purified according to method 3; lane 5, a mixture of the proteins in lanes 1 and 2; lane 6, [K237E]EF-TuHis (EF-TuHisK237E) purified according to method 3.

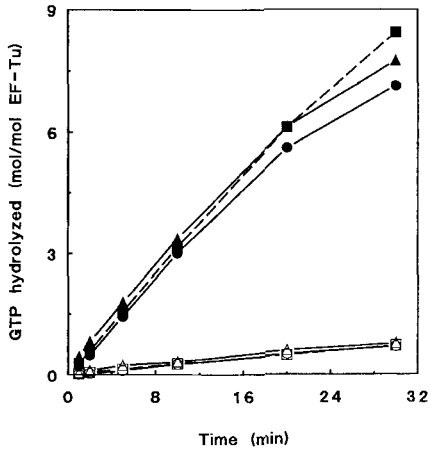

Fig. 4. GTPase activity. The rate of hydrolysis of [γ-^{32}P]GTP by EF-Tu (●), EF-TuHis (▲) or [K237E]EF-TuHis (■) was measured in the presence (closed symbols) or absence (open symbols) of 50 μM kirromycin. Aliquots were withdrawn from the reaction mixture at the times indicated and the $^{32}P_i$ released was determined as described in Materials and Methods.

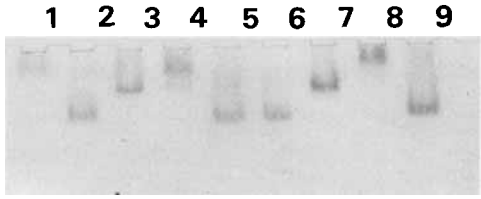

Fig. 5. Analysis of complex formation between EF-TuHis·GTP, [K237E]EF-TuHis·GTP or EF-Tu·GTP and either kirromycin or Xaa-tRNA. Protein bands were visualized by staining with Coomassie Brilliant Blue. Lane 1, EF-TuHis·GTP; lane 2, EF-TuHis·GTP and Xaa-tRNA; lane 3, EF-TuHis·GTP and kirromycin; lane 4, [K237E] EF-TuHis·GTP; lane 5, [K237E]EF-TuHis·GTP and Xaa-tRNA; lane 6, [K237E]EF-TuHis·GTP and Xaa-tRNA, in this case a zone of 32 μM Xaa-tRNA was applied; lane 7, [K237E]EF-TuHis·GTP and kirromycin; lane 8, EF-Tu·GTP; lane 9, EF-Tu·GTP and Xaa-tRNA.

(Fig. 2). Isoelectric focusing revealed that EF-TuHis indeed has an altered isoelectric point (Fig. 3). This experiment further showed that the EF-TuHis was not contaminated with detectable amounts of wild-type EF-Tu. After storage the purified protein started to form aggregates, resulting in a decrease of activity. One of the disadvantages of this method is that the elution takes place close to the isoelectric point of EF-Tu. A second method was therefore tested, which involves the elution of the proteins with an imidazole gradient [21, 22] under physiological conditions. At higher concentrations, the imidazole effectively competes with EF-TuHis for the binding to Ni^{2+}. After applying the cell extract, the Ni^{2+}-nitriloacetic-acid column was washed with buffer A at pH 8.0, containing 0.8 mM imidazole. A low concentration of imidazole is believed to prevent the non-specific binding of a substantial fraction of proteins. Elution with 8 mM imidazole not only removed an important part of the contaminating proteins but also a considerable amount of the EF-TuHis protein. After elution with 40 mM imidazole, the recovery of EF-TuHis was approximately 50%. Analysis of the different fractions by isoelectric focusing revealed that the purified protein still contained wild-type EF-Tu (Fig. 3).

The best results were obtained with the third method, combining the methods mentioned above. The Ni^{2+}-nitriloacetic-acid column was washed extensively with buffer A at pH 8.0 to remove the unbound proteins, whereafter the pH was lowered to 7.0. The EF-TuHis protein was finally eluted from the column with buffer A at pH 7.0, containing 80 mM imidazole, yielding a protein of more than 95% purity (Fig. 2A), without detectable traces of wild-type EF-Tu (Fig. 3). The overloaded sample in lane 7 (Fig. 2A) shows a minor contamination of high-molecular-mass proteins that cross-react with the polyclonal antibodies as can be seen in lanes 1 and 2 (Fig. 2B). This might be due to a trace contaminant in the wild-type EF-Tu preparation used for immunogenesis, since other polyclonal antibody preparations did not reveal these bands. The EF-TuHis protein was stored in buffer A containing 50% (by vol.) glycerol and 20 μM GDP at −20°C.

Functional analysis of the EF-TuHis

In-vitro *analysis*

To investigate whether the His tag influences the enzymic activity of EF-Tu, we first analysed the interactions of EF-TuHis with different components of the elongation cycle *in vitro*. The GDP-binding activity of purified EF-TuHis was found to be at least 80%, the wild-type EF-Tu was found to be 90% active in the same experiment. Also the intrinsic GTPase activity of EF-Tu was not affected by the presence of the His tag and was found to be stimulated by the antibiotic kirromycin in the same way as wild-type EF-Tu (Fig. 4). It should be noted that apparently no non-specific guanosine-5′-triphosphatases were co-isolated by this one-column purification. The activity of EF-Tu to form complexes with Xaa-tRNA or kirromycin was determined using a band-shift assay under native conditions. Complex formation can be observed as a consequence of a difference in electrophoretic mobility compared to that of the free protein [32, 39]. In Fig. 5 it can

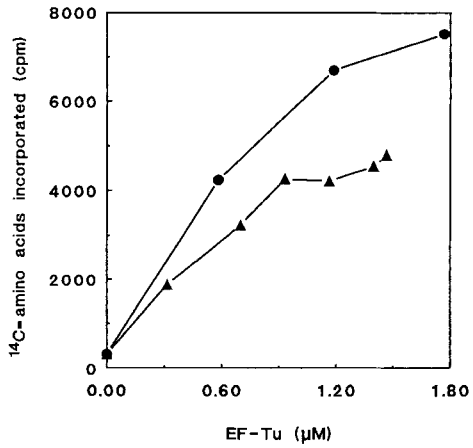

Fig. 6. *In-vitro* translation assay. The translation of a run-off transcript (see Materials and Methods) was measured by studying the incorporation of ^{14}C-labelled amino acids as a function of the concentration of EF-TuHis (▲) or wild-type EF-Tu (●).

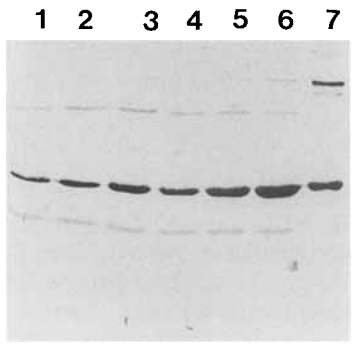

Fig. 7. Expression of EF-TuHis in strain PM1455. Total protein samples of PM1455 cells transformed with the plasmid pKECAHis, taken before and after induction with isopropyl-thio-β-D-galactoside, were analyzed by Western blotting. The blot was analyzed with antibodies raised against the wild-type EF-Tu. Lane 1, strain PM1455 at A_{660} 0.25; lane 2, strain PM1455 after additional growth for 2 h; lanes 3–6, PM1455 carrying pKECAHis (3), after additional growth of 2 h in the absence of isopropyl-thio-β-D-galactoside (4), after 2 h of induction with isopropyl-thio-β-D-galactoside (5), after 4 h of induction with isopropyl-thio-β-D-galactoside (6); lane 7, purified EF-TuHis.

be seen that EF-TuHis is capable of stable complex formation with kirromycin and with Xaa-tRNA. The formation of the complex leads to the same shift of the protein band, as is seen for the wild-type EF-Tu (Fig. 5). The small difference in electrophoretic mobility in lanes 1 and 8 (Fig. 5) can be explained by the difference in charge between wild-type EF-Tu and EF-TuHis.

Evidence for the participation of EF-TuHis in the elongation cycle was obtained using an *in-vitro* translation assay. In this experiment the translation of a run-off transcript of the pT7.1TufB plasmid (see Materials and Methods) was measured by studying the incorporation of a mixture of ^{14}C-labelled amino acids. As can be concluded from Fig. 6, EF-TuHis participates in the translation and behaves in a similar way to wild-type EF-Tu when either protein is added to an EF-Tu-free S30 extract. The extent of amino-acid incorporation was comparable for both proteins and within the error limits observed in a series of independent experiments performed with wild-type and mutant EF-Tu.

In-vivo *analysis*

The preceding results indicate that EF-TuHis is able to sustain the whole elongation process *in vitro*. To demonstrate that EF-TuHis is also active *in vivo*, experiments were performed with the kirromycin-resistant *E. coli* strain PM1455 [26], harbouring only one active *tuf* gene encoding EF-TuAr, an EF-Tu with a low affinity for the antibiotic. Cells of this strain harbouring the plasmid pKECAHis were tested for induction of expression of EF-TuHis and sensitivity to kirromycin. While a high level of expression of EF-TuHis could be induced by addition of isopropyl-thio-β-D-galactoside (Fig. 7), there was no major growth effect detectable after induction in liquid cultures. The sensitivity of the transformants was tested on solid medium. The strain PM1455 could grow in the presence of 100 μg/ml kirromycin while the transformants could not grow at concentrations higher than 30 μg/ml kirromycin. Particularly striking was the effect when the transformants were grown with 1 mM isopropyl-thio-β-D-galactoside. In this case, 5 μg/ml kirromycin was lethal. We conclude that even the presence of small amounts of EF-TuHis, due to the leakage of the *tac* promoter, leads to sensitivity to the antibiotic. The overproduction of plasmid-borne EF-TuHis (Fig. 7) results in a higher sensitivity to kirromycin. Since kirromycin is known to immobilize EF-Tu on the ribosome, thus blocking the translation process, the change in phenotype of the transformants from resistant to sensitive indicates that EF-TuHis is participating in the elongation cycle *in vivo*.

Mutant protein [K237E]EF-TuHis

Overproduction and purification

The expression of the gene encoding [K237E]EF-TuHis could be induced to a high level without considerable growth problems, thus facilitating the purification in preparative amounts. The presence of the mutated codon was confirmed by double-strand sequencing [23] of the plasmid pKECAHis-K237E. The isolation of the [K237E]EF-TuHis (according to method 3, see Materials and Methods) resulted in a mutant protein preparation of at least 90% purity. Fig. 3 shows that the isoelectric point of EF-TuHis is affected by the substitution Lys237→Glu. It is also important to notice that in this mutant-protein preparation no contaminating traces of wild-type EF-Tu were found (Fig. 3). The mutant protein was stored in buffer A containing 50% (by vol.) glycerol and 20 μM GDP at −20 °C.

In vitro *analysis*

Analysis of the enzymic activity of [K237E]EF-TuHis demonstrated that the mutation does not have an apparent effect on the GDP-binding activity of EF-TuHis, nor on its intrinsic GTPase activity. As found for EF-TuHis, the GTPase of the mutated factor can be triggered by the antibiotic kirromycin (Fig. 4), thus showing no major difference with wild-type EF-Tu. Results obtained with gel electrophoresis under native conditions (Fig. 5) show that complex formation with Xaa-tRNA causes the same band shift for [K237E]EF-TuHis as for EF-TuHis and wild-type EF-Tu, indicating that [K237E]EF-TuHis can form a ternary complex. The minor band in lane 5 (Fig. 5) is an impurity of the [K237E]EF-TuHis sample that also remains visible when run in a comigrating zone of 32 μM

Xaa-tRNA without any further interference. In addition, the mutant protein can form a stable complex with the antibiotic kirromycin, as can be observed for EF-TuHis.

DISCUSSION

The purification of mutated EF-Tu species is often a crucial problem since cellular growth requires a functional EF-Tu. The common procedure to obtain mutated protein, i.e. overproduction from a plasmid in *E. coli*, leads however to a heterogenous population: chromosome-borne, native EF-Tu and plasmid-borne, mutated EF-Tu. For the characterization of the mutation it is essential to separate these molecules, since synergistic actions of different EF-Tu species, may occur *in vivo* [35, 36] and *in vitro* [17]. The purification methods available so far depend on biological properties of EF-Tu and are relatively time consuming. In this report we describe a procedure based on Ni^{2+}-nitriloacetic-acid affinity chromatography that allows the rapid purification of native C-terminally tagged EF-Tu proteins free of wild-type EF-Tu.

The three elution methods tested clearly show that the use of a pH gradient results in a highly purified protein without detectable amounts of wild-type EF-Tu. However, the purified protein shows loss of activity. Alternatively, the use of imidazole-containing buffers results in a protein preparation of lower purity. High purity could only be achieved with losses of approximately 50% of EF-TuHis and traces of wild-type EF-Tu are still present. This indicates that the imidazole concentration during the binding to Ni^{2+} and the washing steps is critical. Probably, the strength of the binding to Ni^{2+} is affected by the availability of the histidine residues. To overcome these problems we have combined both methods. The majority of contaminating proteins are eluted by lowering the pH, whereafter the His-tagged protein is eluted at a fixed imidazole concentration, resulting in a simple and fast purification procedure. The purified EF-TuHis is more than 95% pure and free of wild-type contamination (Fig. 3).

The extension at the C-terminus neither influences the GDP-binding capacity of EF-Tu, nor the intrinsic GTPase activity (Fig. 4). In addition, the interaction with the antibiotic kirromycin is not affected, as shown by the stimulation of the GTPase activity (Fig. 4) and by the retardation gel electrophoresis (Fig. 5). Further analysis shows that EF-TuHis is fully active in the binding of Xaa-tRNA as established *in vitro* by a band-shift assay under native conditions (Fig. 5), in contrast to wild-type EF-Tu which shows a variable activity in binding Xaa-tRNA [37] suggesting a conformational heterogeneity in the EF-Tu preparation. It was also shown that EF-TuHis participates in the elongation process *in vitro* (Fig. 6) in a similar way as the wild-type EF-Tu. The activity of EF-Tu seems only slightly affected by the tag, as is suggested by the somewhat lower incorporation rate found for EF-TuHis (Fig. 6). This effect might indicate a slightly disturbed interaction with the ribosome [38].

The results of the *in-vitro* experiments are supported by the experiments performed with the kirromycin resistant strain PM1455. The over-expression of EF-TuHis in this strain alters the phenotype from resistant to sensitive, implying that EF-TuHis is indeed able to function in the translation process *in vivo*. Therefore, the removal of the histidine residues is not necessary. We conclude that the purification method we described here is simple and fast and very suitable for the isolation and characterization of mutated elongation factors. Since the histidine tag at the C-terminal end of EF-Tu appears not to affect essential interactions during protein synthesis, the immobilization of EF-TuHis on the Ni^{2+}-nitriloacetic-acid column may serve various purposes as a functional affinity column.

The structural implications of the EF-Tu Xaa-tRNA interaction are still not well understood. The study of mutated EF-Tu species may, therefore, contribute to the elucidation of the molecular basis of this interaction. One of the amino acids that might contribute to the binding of Xaa-tRNA is Lys237, a strongly conserverd residue both in prokaryotic EF-Tu and eukaryotic EF-1α species. Cross-linking studies have shown that this residue can be linked to the 3′ end of A-site-bound tRNA [10]. Furthermore, the binding of Xaa-tRNA protects this residue from chemical modification [6]. For this reason the Lys237 was substituted by a negatively charged amino acid. The results presented in Fig. 5 show that [K237E]EF-TuHis is able to form the ternary complex, but further experiments are in progress to characterize the protein in more detail.

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