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Dynamics in electron transfer protein complexes

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Chapter 1

Introduction

Protein - protein interactions

Protein-protein interactions perform an integral role in many biological processes. Examples include the interactions involved in converting extracellular signals into appropriate cellular responses (signal transduction)[1,2], the control of gene expression by interactions between transcription factors[3,4], the inhibition of enzymes by intracellular inhibitor proteins[5-8], and the maintenance and regulation of the cytoskeleton[9]. Disruption of protein–protein interactions can cause diseases related to these functions. The study of protein interactions is important because it can lead to the development of new drugs[10] that can specifically modulate protein interactions, instead of targeting a protein's active site. Protein interactions are studied by experimental and theoretical methods, at an ever increasing pace. The strength and duration of the protein interaction is correlated with its function. The kinetics of protein-protein association and dissociation are of major biological and biophysical interest. The affinity between two proteins is a thermodynamic property and can be described by their dissociation constant (K_d). K_d is equal to k_{off}/k_{on}, where k_{off} and k_{on} are dissociation and association rate constants, respectively. Observed values of dissociation constants cover a wide range from 10^{-2} to 10^{-16} M [11,12].

In general, protein-protein complexes can range from static to transient. Static complexes are characterized by a slow dissociation rate, and the partners in the complex usually bind strongly in a single, well-defined orientation. The dissociation constant in these complexes can be as low as 10^{-15} to 10^{-16} M [12,13]. The proteins in these complexes selectively recognize each other and avoid interactions with other cellular components. Examples include complexes of antigens and antibodies as well as enzymes and inhibitors. In these complexes, tight binding is required to completely inhibit the activity of antigen or enzyme.

In contrast, transient complexes form when a high turnover is required, such as in signal transduction cascades or electron transfer chains. Electron transfer reactions are involved in many biological processes such as photosynthesis, respiration and a range of other metabolic processes. Such complexes are characterized by low binding affinities, with K_d values on the order of µM - mM [14], and lifetimes on the millisecond time scale. According to Marcus theory of electron transfer[15,16], rate of electron transfer (k_{et}) drops

exponentially with the increase in distance between the redox centers. As a consequence, formation of a specific complex is necessary for an efficient electron transfer. Although the interactions of these partners are tuned to be of low affinity, the specificity should be sufficient for electron transfer to occur efficiently. In general, these proteins must participate in transient interactions with several partner proteins. Therefore, the protein must necessarily recognize and interact with partners with distinct surface properties and structures limiting the specificity of any one interaction. The transient protein complexes have high association and dissociation rates. The high association rate facilitates the complex formation and high dissociation rate ensures high turn over. A relative rotation of as little as a few degrees or a relative translation by a few Ångströms is sufficient to break all specific interactions between the two proteins. Most of the present knowledge about transient complexes comes from electron transfer complexes. However the means by which electron transfer proteins achieve the paradoxical requirements of low affinity for partners, yet adequate specificity and electron transfer reactivity, are still poorly understood. The biological mechanism of how the electron transfer proteins manage to achieve high turnover is an important question driving the work in this thesis. Recent work has highlighted the role of the encounter complex in this matter.

Encounter Complex

The biological message between the proteins is transferred via physical interactions through their binding sites. In order to convey the message, the proteins must approach each other by diffusion and bind through specific surface patches. As the patch constitutes just a small fraction of the total protein surface, only a small fraction of the collisions will bring proteins in the proper orientations resulting in low association rates. However, in many of the biological processes like electron transfer and signal transduction, a quick transfer of the message is crucial, which requires fast association and dissociation of the proteins. The fast association of the proteins can be achieved by increasing the life time of the collision and reducing the surface area searched by the proteins to find the interface. This is achieved by the formation of the encounter complex[17] prior to the formation of the well defined complex.

The association of proteins to form a complex is a multistep process, which starts by random collisions of the individual proteins. The proteins first associate to form an encounter complex. This part of the process is diffusion controlled and dominated by non-specific electrostatic interactions[6,18]. These interactions keep the macromolecules in proximity for a prolonged time, allowing a more extensive two dimensional search of the surface of the partner by translational and rotational movements. In the encounter complex, the proteins can reorient their interaction patches, which is required for formation of the bound complex. The encounter complex either proceeds towards the final complex or dissociates again (Figure 1.1). The well-defined complex is dominated by short range interactions like van der Waals forces and hydrogen bonding. The dominant role of electrostatic forces in the initial stage of complex formation is a consequence of their long-distance nature, in contrast to the short-range forces that are responsible for specificity.

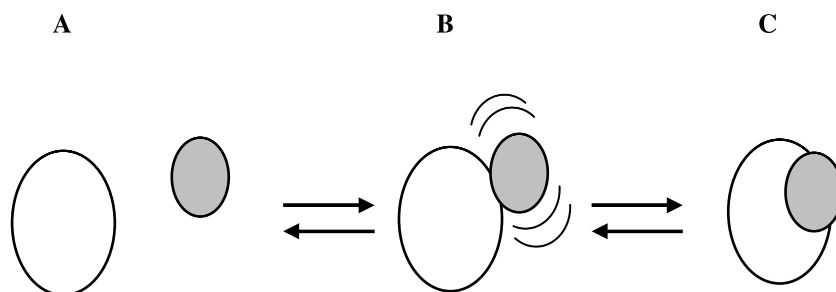


Figure 1.1: Model for protein complex formation. Free proteins (A) associate to form encounter complex (B) consisting of multiple protein orientations which leads to the formation of single orientation specific complex (C).

The encounter complex has been visualized in a number of protein-protein ([19,20], this work, chapter 4,5 and 6) and protein-DNA[21,22] complexes. Experimental and theoretical studies have provided evidence that transient nonspecific encounter complexes play an important role in protein binding and function. The nature and in particular the fraction of the encounter complex differs between complexes, depending on the biological role of the complex. Tight complexes are likely to have the equilibrium shifted towards the productive complex and exchange between productive and encounter complex could be

slow. For relatively weak complexes, the encounter complex represents a remarkable fraction of the complex ([19,23], this work, chapter 4,5 and 6) and may be in fast exchange with the productive complex, maintaining the right balance between specificity and fast association. In some cases, the complex can have a larger fraction of the encounter complex than the specific complex ([19,24,25], this work, chapter 5) or even be a pure encounter complex[26-30]. Mutations in the interface may shift the equilibrium between the encounter complex and the specific complex ([31,32], this work, chapter 5 and 6).
Despite the importance of encounter complexes, a reliable way to determine their relative populations or to visualize the structures is lacking, largely because of their diffusive nature. The complete picture of the complex formation can only be achieved by studying both the well-defined, productive complex and the dynamic encounter complex.

NMR methods to study protein complexes

In recent years NMR has proven to be a very useful technique to study the protein complexes. Various NMR methods have been developed to investigate the protein structure, binding and dynamics in solution. The word dynamics is used to describe the protein motions including side chains and domains. Dynamics also refers to the movement of one protein around the other in transient complexes.

Chemical shift perturbation analysis

One of the commonly used NMR methods used to probe protein-protein interactions is the chemical shift perturbation analysis[33]. It helps to delineate the binding interface and to estimate the association and dissociation rates of the protein complexes[34]. In this method HSQC or TROSY spectrum of ^{15}N labelled free protein is usually recorded. Each peak in the spectrum corresponds to a specific amide group in the protein. Then the unlabelled partner is titrated into the protein solution and the NMR spectrum is recorded again. Binding of the partner protein changes the chemical environment of nuclei in the binding site. The nuclei at the interface feel chemical perturbations, which result in chemical shift changes. The size of the changes can be fitted to get the binding

constant of the two proteins. The average size of the shift changes also provides information on the dynamics of the protein complex[29,30]. The more dynamic the complex, the smaller the chemical shift changes are. In this way the average size of the chemical shift changes reports on the relative population of the encounter complex and the specific complex. A specific complex has a specific orientation and is stabilized by short-range interactions like hydrogen bonding and salt bridges, resulting in large chemical shift changes. The encounter complex exists in multiple orientations and it is assumed that at least a single solvation layer remains. As a consequence, the chemical shift changes are small and averaged over all orientations. Chemical shift perturbations fail to provide accurate information on the binding interface of the proteins which undergo large conformational changes upon complex formation. In such cases the conformational changes can result into chemical shift changes of nuclei far from the interface. However, the method is very useful to study the interface of transient complexes which do not undergo large conformational changes.

Paramagnetic relaxation enhancement

Paramagnetic relaxation enhancement (PRE) is another widely used NMR method to determine structures and study the dynamics in protein complexes[19,20]. Paramagnetic effects arise from an unpaired electron on a metal ion or stable organic radical. The unpaired electron increases the relaxation rate of the nuclei in its vicinity, due to the large magnetic moment of the unpaired electron. The effect depends on the sixth power of distance (r^{-6}) between the nucleus and the unpaired electron. PRE provides unique long range distance information from 10 Å to 35 Å[35]. A large number of metalloproteins contain intrinsic paramagnetic centers and have been studied by this method. This method has been extended to proteins that do not contain metals by the introduction of a paramagnetic center on the protein surface. A number of paramagnetic centers like nitroxide spin labels and metal chelating tags are in use to study protein interactions. These probes can be covalently attached to the protein surface via cysteine residues. The paramagnetic center is attached to one protein and the relaxation rates of the nuclei in the other protein are measured. These PREs are then converted into distance restraints which are used to solve the structure of the protein complex[19,36]. PRE has also proved to

be a very useful technique to visualize the encounter complex. As the effect of spin label is proportional to r^{-6}, nuclei that are very close to the paramagnetic center are strongly affected. Therefore, this approach can be used to detect orientations that represent only a few percent of the complex, as has been demonstrated for protein-protein[19,20] and protein-DNA[21,22] complexes as well as macromolecular self-association[37,38], allostery[39] and state equilibria[40]. In this work, for the first time the complete surface area sampled during the encounter complex has been mapped out, by applying spin labels at different positions on the protein surface and measuring the effects on the other protein.

PRE is a very useful technique to detect minor orientations of the complex. However, it is impossible to distinguish individual orientations of the encounter complex by this method, because the NMR spectra give an average of free proteins, all orientations of the encounter complex and the specific complex.

Computational methods to study protein-protein interactions

There is a wide spectrum of computational methods for prediction, identification and characterization of protein-protein interactions[41]. These methods have been developed for the identification of binding site on the protein surface[42,43], the calculation and analysis of association rates[44,45], structure calculation of the protein complexes[41] and to study the dynamics of the protein complexes[46,47]. Besides providing intrinsically valuable structural biology information, these methods can help to explain binding affinities and specificities, the nature of the binding free energy funnel and effects of mutations.

Many docking methods are available to predict the structures of protein complexes if the structures of the individual proteins are known. Most of these docking methods aim to predict the atomic model of a complex by maximizing the shape and chemical complementarity between a given pair of interacting proteins[48,49]. Docking strategies usually rely on a two-stage approach: they first generate a set of possible orientations of the two docked proteins and then score them in the hope that the true complex will be ranked highly. The hypothesis underlying these docking predictions is that the structure of a complex is the lowest free energy state accessible to the system. These methods

differ in protein representation, in the scoring of different configurations and in the search for the best solutions. Although docking methods are not sufficiently accurate to predict whether or not two proteins actually interact with each other, they can sometimes correctly identify the interacting surfaces between two structurally defined subunits[50]. The docking is often applied in concert with experimental techniques, including site-directed mutagenesis[51], amide hydrogen-deuterium exchange[52] and NMR spectroscopy[53,54]. Methods that are able to work with comparative protein structure models[55] instead of experimentally determined subunit structures would extend the applicability of docking to many more biological problems, but would probably have poorer performance. These docking methods, however, are only applicable to the small fraction of complexes for which the structures of the two interacting proteins are known. The kinetics of macromolecular association events are of interest because of their potential biological importance. A number of enzymes have evolved to such an extent that the rate-limiting step of their catalytic cycle is their association with their substrates[56]. Because association is the process that places an upper limit on the overall efficiency of these enzymes, simulations that can address this process may, in addition to providing insight into the factors controlling the process, also suggest ways to accelerate (or otherwise modulate) the process so as to produce more efficient enzymes. Brownian Dynamics and Monte Carlo simulations have been used to simulate the encounter complex, and to study the kinetics in protein complexes. The proteins are treated as rigid bodies and the interactions are defined either at residue level or at atomic level. These simulations have been successfully applied to protein complexes which do not undergo large conformational changes on association.

In Brownian Dynamics simulations, the two proteins are first separated by a large distance. Then, a series of simulations is performed where diffusion of one of the proteins is simulated until it either binds to the other protein or escapes to some larger distance. The association of the two proteins is defined by monitoring the distances between certain pairs of atoms known to be adjacent to each other in the final complex. Association is assumed when one or more of these atom pairs come within a specified distance (usually 6-7 Å). This is equivalent to assuming that once the atoms reach this separation that subsequent association is completely assured. By performing many (thousands of) simulations, statistically robust estimates of the probability that

association will occur can be calculated. In Monte Carlo simulations, the electrostatic potentials of the proteins are calculated on the basis of the structure, and the atomic charges of the proteins are defined. The proteins are confined to a sphere depending on the size of the proteins and one protein is allowed to translate and rotate in the electrostatic potential of the other protein. At each step the electrostatic interaction energy is calculated and moves are accepted or rejected by applying certain criterion which depends on the energy. An exclusion grid is used to avoid steric overlap between the proteins and the association of the two proteins is determined by defining a minimum distance between the proteins. The structures are saved at multiple steps and this procedure gives a Boltzmann distribution of orientations based on the electrostatic interaction energy. Earlier studies have shown that the encounter complex is dominated by the electrostatic interactions[6,18]. By specifically defining these interactions between the two proteins, it is possible to simulate both the encounter complex and the specific complex or the encounter complex only.

NMR data combined with the docking can be used to solve the structures of the protein complexes. The experimental methods need to be combined with the theoretical approaches to achieve greater accuracy, coverage, resolution and efficiency than any of the individual methods. Several approaches have been proposed so far to visualize the encounter complex by combining modeling and PRE data, including explicit ensemble refinement[20], Brownian Dynamics simulations[23], and empirical ensemble simulations[26,32]. The combination of the experimental data and the theoretical simulations can provide much more information about the complex formation, which cannot be obtained by the individual methods.

Proteins and complexes studied

The proteins studied in this research are involved in the biological electron transfer. Long-range electron transfer reactions involving cytochromes (a class of haem-containing proteins) are prevalent in energy producing biological processes such as photosynthesis and respiration. Respiration, in particular, is the process by which dioxygen is ultimately reduced to water, producing energy-rich adenosine triphosphate (ATP) along the way. The machinery of the respiratory electron transport chain (Figure

1.2) is located in the inner mitochondrial membrane of eukaryotic cells or in the plasma membrane of respiring bacteria.

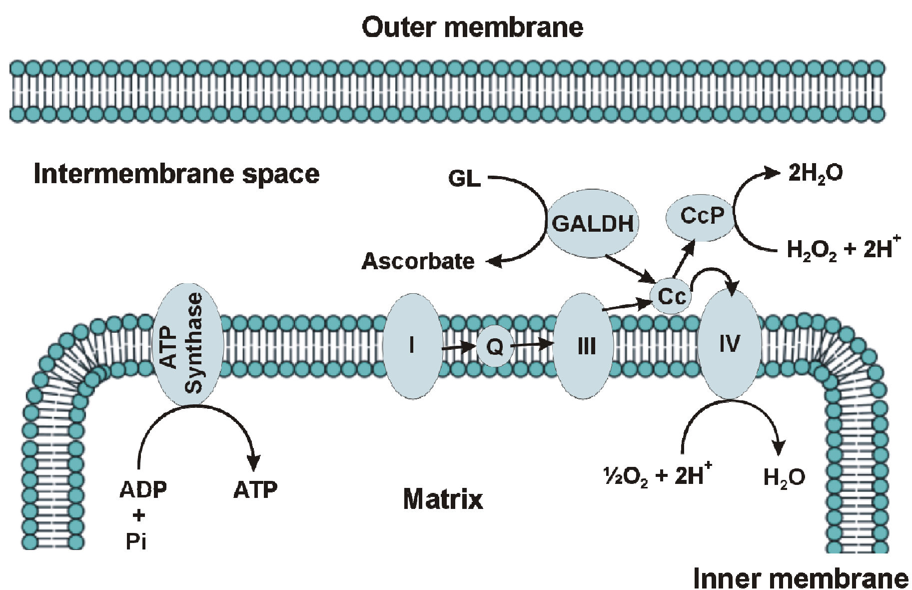


Figure 1.2: Schematic representation of electron transport in plant mitochondria.

Cytochrome *c*

Cytochrome *c* (Cc) is a small haem protein found loosely associated with the inner membrane of the mitochondrion. Cc is a positively charged protein with isoelectric point in the range of 10.0 to 10.5. Cc is a highly soluble protein with a solubility of about 100 g/L. Cc is a highly conserved protein across the spectrum of species, found in plants, animals, and many unicellular organisms. Because of its ubiquitous nature and sequence homology, Cc has been used as a model protein for molecular evolution. A number of high resolution X-ray and NMR structures of both ferrous[57,58] and ferric[59-61] Cc have been reported. Cc has a nearly spherical shape with 5 α-helices and a short β-strand (Figure 1.3A). It is a small protein with a molecular weight of about 12 kDa, comprising 100 to 108 amino acid residues with a *c*-type haem group. A haem is a prosthetic group comprising an iron atom in the center of a large heterocyclic organic

molecule called a porphyrin. In *c*-type cytochromes the haem is covalently attached to the protein by two thioether bonds involving sulphydryl groups of cysteine residues. The iron atom has six coordination positions of which four are occupied by the nitrogen atoms of the pyrole rings. In Cc the two addional ligands are the $N^{\varepsilon2}$ atom of a histidine and the S^{δ} atom of a methionine (Figure 1.3B). The iron ion interconverts between the Fe^{2+} (reduced) and Fe^{3+} (oxidized) states in electron-transfer processes. The iron is diamagnetic in reduced and low spin paramagnetic in oxidized state. The transition of Cc between the ferrous and ferric states makes it an efficient biological electron transporter and it plays a vital role in cellular oxidations in both plants and animals.

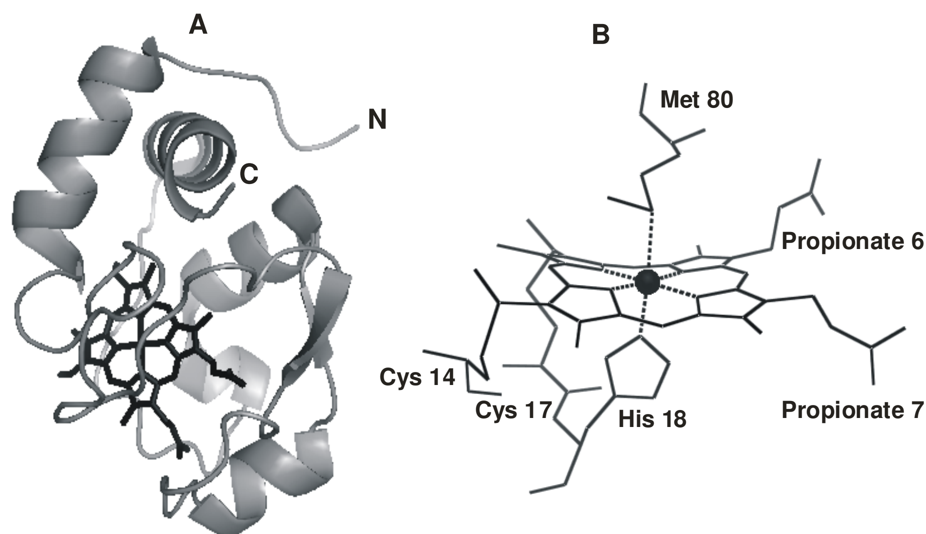


Figure 1.3: Three-dimensional structure of yeast Cc. **(A)** Overall view of the protein. Cc is shown as ribbons and the haem is shown in sticks. **(B)** The view of the haem binding site showing the axial ligands, His 18 and Met 80. The two cysteine residues, Cys 14 and Cys 17, covalently bound to the haem group, and haem propionates are also shown. The molecular coordinates were taken from the crystal structure of the oxidized Cc (PDB entry 2YCC[59]).

Cc is primarily known for its function in the mitochondria as a key participant in the life-supporting function of ATP synthesis. It is an essential link in the electron transport chain through which cells perform the controlled "burning" of glucose and capture much of that released energy by storing it in ATP, the cell's primary energy distribution

molecule. Its main function in cellular respiration is to transport electrons from cytochrome *c* reductase (Complex III) to cytochrome *c* oxidase (Complex IV) embedded in the inner membrane of the mitochondrion[62]. Thus, Cc undergoes repetitive cycles of oxidation and reduction, but it does not bind oxygen. In yeast, Cc has other physiological partners such as cytochrome *c* peroxidase and cytochrome b_2. It is generally regarded as a universal catalyst of respiration, forming an essential electron-bridge between the respirable substrates and oxygen. Interestingly, Cc is also a key factor in apoptosis, a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage[63]. The release of mitochondrial Cc into the cytoplasm stimulates apoptosis and is commonly used as an indicator of the apoptotic process in the cell.

L-galactono-γ-lactone dehydrogenase

L-galactono-γ-lactone dehydrogenase (GALDH) is a mitochondrial flavoenzyme that catalyzes the final step in the biosynthesis of vitamin C (L-ascorbic acid) in plants. It belongs to the family of closely related flavin dependent aldonolactone oxidoreductases[64,65], which catalyze the terminal step in the biosynthesis of ascorbate and its analogs. GALDH catalyzes the oxidation of L-galactono-1,4-lactone to L-ascorbate using Cc as an electron acceptor[66].

GALDH has been extracted for the first time from cauliflower florets[67] and since then has been isolated from the mitochondria of a number of other plants, including white and sweet potato[68,69], spinach[70], cauliflower[71], sweet pepper[72], and kidney bean[73]. GALDH from *Arabidopsis thaliana* has been expressed in *Escherichia coli* and characterized[66]. The GALDH enzymes isolated from various sources have the same molecular mass (56 kDa) and share approximately 80–90% sequence identity. Although GALDH has been isolated from various sources, no information is available about the 3D structure or active site of this enzyme. A recent study on the RNA interference silencing of GALDH from tomato revealed the importance of GALDH for plant and fruit growth[74]. A severe reduction in GALDH activity can be lethal to the plant.

GALDH is localized in the mitochondrial intermembrane space where it shuttles electrons into the electron transport chain via Cc. Most aldonolactone oxidoreductases

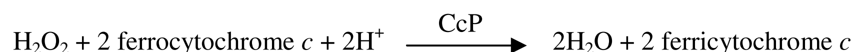
contain a histidyl-FAD as a covalently bound prosthetic group. GALDH lacks the histidine involved in covalent FAD binding. It contains a leucine (Leu56) at this position, indicating that the flavin cofactor is noncovalently bound to the protein[66].

L-ascorbic acid (vitamin C) is an important antioxidant, redox buffer and enzyme cofactor for many organisms. Plants and most animals can synthesize L-ascorbic acid, but humans and other primates have lost this ability. Fruits and vegetables are the main dietary source of vitamin C for humans. L-ascorbic acid is particularly abundant in plants, in which it protects cells from oxidative damage resulting from abiotic stresses and pathogens and functions as a cofactor for a number of enzymes[75].

The mechanism of L-ascorbate production by GALDH involves two half-reactions. In the reductive half-reaction, the oxidized flavin cofactor is converted to the hydroquinone state by the L-galactono-1,4-lactone substrate. The two-electron reduced enzyme is then re-oxidized in the oxidative half-reaction by Cc. This half-reaction involves two subsequent one-electron steps and the formation of a flavin semiquinone radical.

Cytochrome *c* peroxidase

Cytochrome *c* peroxidase (CcP) is a water-soluble haem-containing enzyme of the peroxidase family that takes reducing equivalents from Cc and reduces hydrogen peroxide to water[76]:



$$H_2O_2 + 2\text{ ferrocytochrome } c + 2H^+ \xrightarrow{\text{CcP}} 2H_2O + 2\text{ ferricytochrome } c$$

CcP can react with hydroperoxides other than hydrogen peroxide, but the reaction rate is much slower than with hydrogen peroxide. CcP is one member of a large family of haem peroxidases. These enzymes link reduction of hydroperoxides (i.e. ROOH to ROH + H_2O) to oxidation of an electron donor. The reaction mechanism with peroxide is similar for all of the haem peroxidases, and the haem binding pocket is highly conserved.

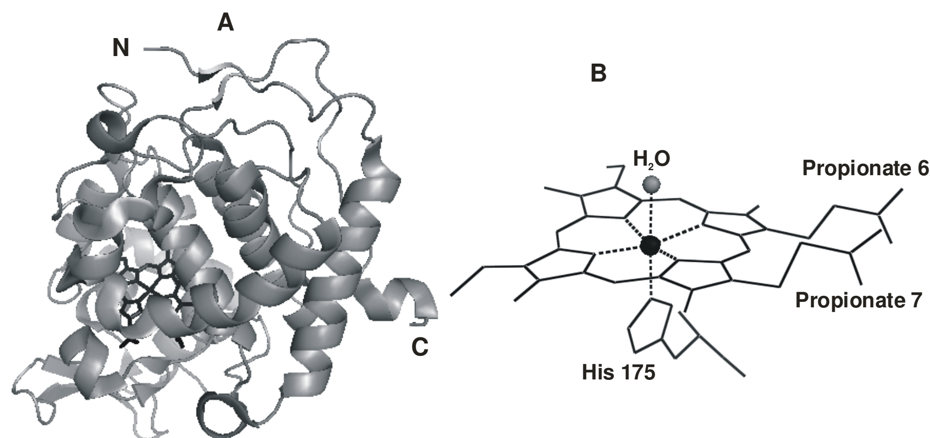


Figure 1.4: Three-dimensional structure of yeast CcP. **(A)** Overall view of the protein. CcP is shown as ribbons and haem is shown in sticks. **(B)** The view of haem binding site showing the axial ligand His 175 and haem propionates. The molecular coordinates were taken from the crystal structure of the resting state CcP (PDB entry 1ZBY[77]).

CcP was discovered in and purified from bakers' yeast by Altschul, Abrams, and Hogness in 1940[78]. CcP is an acidic protein present in the intermembrane space of mitochondria, with a reported isoelectric point between 4.9 and 5.25. The yeast CcP is a monomer of molecular weight 34 kDa, containing 294 amino acids. Unusual for proteins, this enzyme crystallizes when dialysed against distilled water. The enzyme purifies as a consequence of crystallization, making cycles of crystallization an effective final purification step. CcP was the first haem enzyme to be crystallized. Its X-ray structure was solved in 1980 by Poulos and coworkers[79].

CcP does not have a well-defined metabolic role, but seems to be designed to detoxify hydrogen peroxide (H_2O_2) formed in the intermembrane space of mitochrondria during aerobic metabolism. H_2O_2 is formed as a result of incomplete reduction of O_2 to water. The toxicity of H_2O_2 arises from its ability to form free radicals, e.g. hydroxyl radicals, which are highly reactive and can result in cell damage or cell death. H_2O_2 may be removed by catalase in a dismutation reaction or by peroxidase in a process of reduction to water.

CcP contains a single *b*-type haem, burried in a hydrophobic pocket within the protein. The haem is non-covalently bound to CcP and coordinated by the $N^{\varepsilon 2}$ atom of His 175,

the only axial ligand. The sixth coordination position is vacant for binding of the peroxide. In the resting state of the enzyme, the sixth coordination position is occupied by a water molecule.

Although CcP holds little interest from a metabolic perspective, its ability to promote rapid electron transfer from Cc makes it a useful structural model for the more complex electron transfer systems that participate in aerobic metabolism. Moreover, its reaction with peroxide is quite similar to other known peroxidases, and it is widely viewed as a model for this reaction.

Cytochrome *c* – cytochrome *c* peroxidase complex

The Cc-CcP system has been extensively investigated as a model for long-range inter-protein electron transfer[80]. The physiological complex of yeast Cc and yeast CcP is one of the few transient ET complexes for which a crystal structure has been solved[81] (Figure 1.5). The crystal structure shows that the complex is mainly stabilized by van der Waals interactions and a single hydrogen bond between an asparagine (N70) of Cc and a glutamic acid (E290) of CcP. The orientation of Cc in the complex in solution has also been determined by NMR[19]. It has been shown that the Cc-CcP complex is highly dynamic and its crystal structure shows the dominant form observed in solution. The complex formed between Cc and CcP serves as a simple model for the much more elaborate electron transfer complexes of the respiratory electron transport chain. CcP catalyzes the reduction of peroxide to water using two electrons from two molecules of Cc. During the catalytic cycle, peroxide reacts with ferric CcP to form an intermediate called compound ES and a molecule of water[82]. Compound ES retains the oxidizing equivalents of peroxide as oxy-ferryl haem (Fe^{IV}=O) and an indolyl cation radical at Trp 191 ($Trp^{\bullet+}$)[83-87]. The two oxidized sites of compound ES are then reduced by two molecules of reduced Cc to regenerate the ferric CcP and another molecule of water[88,89]. The stoichiometry of Cc:CcP complex formation has been a subject of debate for several years. The crystal structure and the solution structure of the Cc:CcP complex show only one binding site with a 1:1 binding stoichiometry of the two proteins. However, in several studies, it has been proposed that at low ionic strength, the binding of Cc and CcP occurs with a 2:l binding stoichiometry, involving nonoverlapping

binding domains. The formation of a 2:l complex was first demonstrated by size-exclusion chromatography[90,91]. It showed that the first molecule of Cc binds with a high affinity and the second with a much lower affinity. The binding of Cc to the low-affinity binding site appears to affect the steady-state kinetics at low ionic strength[92,93]. Several subsequent reports involving steady-state fluorescence quenching titrations[94], analytical ultracentrifugation[95], and electronic difference spectroscopy[96] led to the conclusion that only a single binding site exists, although an alternative strategy for fluorescence quenching titrations suggested the presence of a second site[91].

Hoffman and co-workers[97-99] have studied the affinity and stoichiometry of binding in complexes between yeast Cc and Zn-porphyrin CcP, and between Zn-porphyrin horse Cc and CcP. Their studies have revealed two distinct binding sites for Cc: A poorly reactive high-affinity site with multiple, overlapping sub-sites for Cc and a highly reactive low-affinity site elsewhere on CcP. They have shown that at low ionic strength (< 50 mM) CcP simultaneously binds two molecules of Cc at two distinct binding sites with widely different affinities and electron transfer activities.

Yi et al.[100] have reported NMR studies of the complex that can be interpreted in terms of two binding sites for the cytochrome. However, the NMR studies by Worrall et al.[101] at high ionic strength showed that the binding occurs in 1:1 stoichiometry and the interaction site in solution in similar to the one in the crystal. The Brownian dynamics simulations by Northrup et al.[102] indicated a high affinity site on the peroxidase corresponding to the one in crystal structure of the complex, and a low-affinity site near Asp 148 of CcP. However, this low-affinity site near Asp 148 of CcP has not been detected in the electrostatic calculations by Gabdoulline and Wade[103].

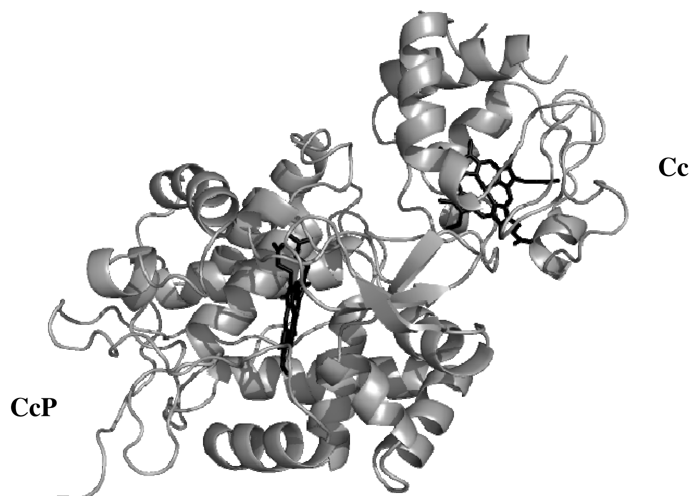


Figure 1.5: Crystal structure of complex between yeast Cc and yeast CcP (PDB entry 2PCC)[81]. The haem groups of both proteins are shown in sticks.

Thesis outline

The aim of the research described in the thesis is to study the dynamics of complexes of Cc with CcP and GALDH in order to elucidate the principles that govern the interactions in transient protein complexes. Emphasis has been given on the role of encounter complex in protein complex formation.

In chapter 2, the interaction of yeast Cc with GALDH and a nine-fold charge mutant has been studied by NMR chemical shift perturbation analysis. The interaction site on Cc for binding with GALDH has been mapped and compared with the complexes of Cc with other proteins. This work is the first structural study on complex of GALDH with its electron acceptor.

In chapter 3, a binding hot spot residue in the weak transient complex of yeast Cc and yeast CcP has been identified and studied by NMR and a double mutant cycle. It represents new and as yet scarce evidence that also in weak and transient complex hot spots of affinity occur.

Chapter 4 describes the characterization of the encounter complex between yeast Cc and yeast CcP using PRE NMR spectroscopy and Monte Carlo simulations. The

conformational space searched by the two proteins in the encounter complex has been mapped out experimentally and a new method to determine the fraction of the encounter complex is proposed.

Chapter 5 describes the effect of interface mutations on dynamics and the relative populations of the encounter complex and the specific complex in yeast Cc-CcP complex. It is demonstrated that the fraction of the encounter complex can be modulated with point mutations in the interface.

Chapter 6 is about the dynamics in horse Cc and yeast CcP complex. The encounter complex has been characterized and the effect of interface mutations on the relative population of the encounter complex and the specific complex has also been studied. It is shown that this non-specific, non-physiological complex can be turned into a specific complex resembling the native yeast complex by a single, conservative mutation.

The results of these studies are discussed in relation to other recent work on the encounter complex in the concluding remarks.