Understanding protein complex formation: the role of charge distribution in the encounter complex

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Chapter 2

Introduction to cytochrome *c* and cytochrome *c* peroxidase

An electron transfer complex

The aim of the research reported in this thesis is to test how critical the charge distribution on protein surfaces is for the protein complex formation. Redox proteins are evolutionary optimized to perform efficient electron transfer, forming transient complexes with a high population of encounter complex. The subject of this thesis is the well characterized complex formed by cytochrome *c* (Cc) and cytochrome *c* peroxidase (CcP) from baker's yeast (*Saccharomyces cerevisiae*). Electrostatic interactions drive the formation to the Cc:CcP complex.[1–5] In this work we modified the charge distribution of CcP and studied how the disturbance of the electrostatic surface impacted on the encounter complex with Cc and on the formation of the active complex. The complex was mentioned in Chapter 1 and the proteins are further introduced here.

Cytochrome *c*

Cytochrome *c* (Cc) was discovered in 1925 by David Keilin.[8] More than one hundred variants are known today and they show a highly conserved sequence among eukaryotes.[9] Cc is one of the main electron transfer (ET) proteins in the eukaryotic respiratory chain, where it functions in the energy production. In this role, it accepts a single electron at a time from cytochrome *c* reductase (cytochrome bc_1, Complex III) to reduce cytochrome *c* oxidase (Complex IV).[10] Cc has additional roles in the regulation of apoptosis as defense mechanism against DNA damage[11] and in baker's yeast (*Saccharomyces cerevisiae*) in the neutralization of oxidative stress caused by hydrogen peroxide, with the aid of cytochrome *c* peroxidase (CcP).[9] The interaction with this enzyme will be discussed further in this thesis.

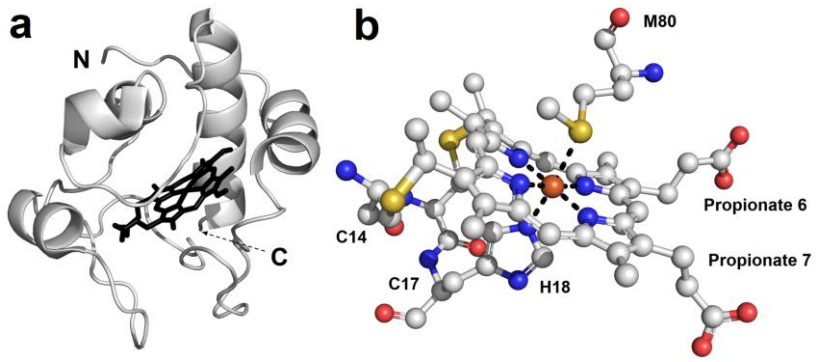

Figure 2.1 Three dimensional structure of Cc. a) Structure of Cc with the haem in sticks. "N" and "C" indicate the two termini of the polypeptide chain. b) Representation of the haem (iron ion in orange) covalently bound to the residues C14 and C17, and axially coordinated to M80 and H18. The molecular coordinates were taken from the crystal structure of the oxidized Cc (PDB entry 2YCC)[6].

In this work the native iso-1 isoform of *S. cerevisiae* Cc was used, the most studied so far.[12] The yeast iso-1 form of Cc is a small, positively charged protein (+6 at pH 6)[13] with 108 amino acids and a haem prosthetic group resulting in a total molecular weight of 12.1 kDa.[4] The native yeast iso-1 Cc harbours a post-translational modification of the residue Lys 72, the N-ε-trimethylation, which blocks the apoptotic activity of the protein.[14] The protein can easily be purified from *S. cerevisiae*[15–17] or from *Escherichia coli*[18,19]. Protein produced in bacteria generally do not have post-translational modifications, thus Cc is not trimethylated in *E. coli*. To obtain a high yield in *E.coli*, Cc is produced in the cytoplasm of the cell along with the haem lyase for the insertion of the haem group into the protein.[20] Cc consists of five α-helices and a small β-strand, folded nearly into a sphere (Figure 2.1a). Its *c*-type haem group is connected to two cysteine residues (14 and 17) by thioether bonds, as part of the conserved with the C-X-Y-C-H sequence.[4] The low-spin iron ion of the haem group is hexacoordinated, equatorially to the four pyrrole nitrogens of the haem and axially to the residue H18 and M80 (Figure 2.1b). The iron ion, which can be reduced Fe(II) or oxidized Fe(III), has magnetic properties that depend on the oxidation state. In the reduced state it is diamagnetic and when oxidized it is paramagnetic.[9] Various high resolution X-ray crystallography and solution NMR structures were published for both the ferrous[21,22] and ferric form.[4,23,24]

Cytochrome *c* peroxidase

Cytochrome *c* peroxidase (CcP) was discovered in the mitochondrial intermembrane space of *S. cerevisiae* by Altschul, Abrams and Hogness in 1940.[25] As previously mentioned, CcP inactivates hydrogen peroxide, reducing it to water, by transferring the electrons accepted from two molecules of Cc. Yeast CcP is a negatively charged protein (-4 at pH 6)[26] with 294 amino acids and a molecular weight of 34.2 kDa. The crystal structure of CcP[27] shows a secondary structure predominantly composed of α-helices and a *b*-type haem at the center, enclosed in a hydrophobic pocket (Figure 2.2a). Contrary to the *c*-type, the *b*-type haems are not covalently bound to the polypeptide chain. The iron of the haem is axially coordinated to the $N_{\varepsilon 2}$ atom of H175 and equatorially to the four pyrrole nitrogens of the haem group. The last coordination position is occupied by a water molecule in the resting state of CcP and available to bind the substrate (Figure 2.2b).[27] During the reaction, CcP goes through several states. In the resting state (RS), the high-spin iron ion Fe^{3+} is pentacoordinated and paramagnetic. When binding hydrogen peroxide, CcP forms the oxyferryl intermediate, called compound I (CpdI),[28,29] in which the iron ion is oxidized to Fe^{4+} and the side chain of Trp191 is oxidized to a cation indole radical.[30–32] One water molecule is released. Each of two molecules of Cc transfer an electron to CcP. The first electron reduces CpdI to form compound II (CpdII) while the second electron restores the RS CcP and releases the second water molecule.[33] For more details on the mechanism of the reaction see chapter III. Similar to Cc, CcP can be purified from *S. cerevisiae*[34–36] or recombinantly overproduced in *E. coli*,[37–42] which yields *apo*-protein. The haem group can be incorporated during the purification process.

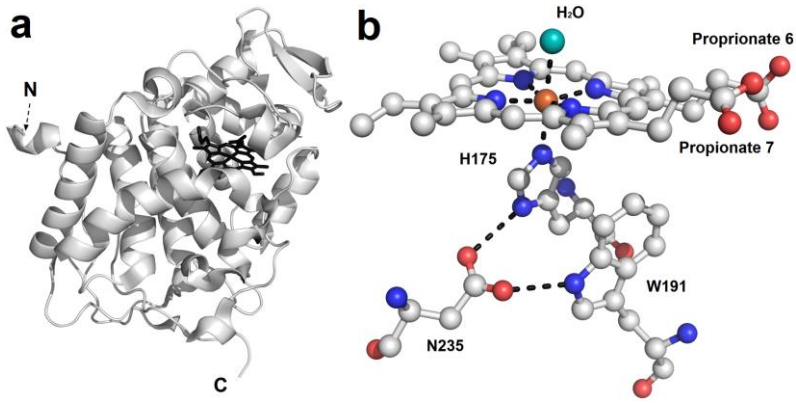

Figure 2.2 Tree dimensional structure of CcP. a) Structure of CcP with the haem in sticks. "N" and "C" indicate the two termini of the polypeptide chain. b) Representation of the haem (iron ion in orange) axially coordinated H175 and to water. The catalytically important W191 and the D235 are also indicated. The molecular coordinated were provided by the crystal structure of resting state CcP (PDB entry 1ZBY)[20].

Cc-CcP complex

The complex formed by Cc and CcP is one of the best characterized ET complexes and extensive literature is available on the subject.[12,43–45] To perform an efficient reaction, the Cc:CcP complex is highly dynamic (see Chapter 1) and the interactions between the two proteins are based on electrostatic interactions between the negatively binding site on CcP and the positively one on Cc (Figure 2.3b).[2,5–7] Despite being a transient complex, a co-crystal structure was reported by Pelletier and Kraut in 1992[1] (Figure 2.3). Although the structure appeared initially stabilized by a single hydrogen bond between N70 of Cc and E290 of CcP[1], other studies suggest the presence of two additional hydrogen bonds: One between Q16 of Cc and A193 on CcP, and a second between K87of Cc and E32 of CcP.[46] Site-directed mutagenesis and biophysical techniques were used to investigate the importance of several residues for the activity of the complex.[43,47–49] Although the crystal structure shows one stereospecific binding site, the stoichiometry of the Cc:CcP complex has been discussed for decades. The 1:1 stoichiometry model was observed under many experimental conditions.[12] On the other hand, several kinetic studies suggested the presence of a second low-affinity binding site[50–52] at which Cc also binds at low salt concentrations (less than 100 mM salt).[53–56] The possible location of the second binding site was investigated by mutagenesis and simulations, suggesting the regions close to the residues D148[5,57] and between D217 and Y39[58,59] as possible interaction sites. A PRE experiment confirmed the presence of a second binding site in 2015.[56] Isotopically labelled CcP and Cc were cross-linked at the crystallographic binding site while free Cc was tagged with a paramagnetic tag. Thus, paramagnetic relaxation was observed on the spectrum of CcP showing that the low-affinity complex consists of an ensemble of minor states sampling two non-overlapping regions of the CcP surface: One between the residues D148, D217,

D33 and E35 and a second region in proximity of the residues E167, D261 and E267. The residues D148 and D217 play an important role for binding at the low-affinity binding site (Figure 2.3).[56]

Different models have been used to describe the Cc and CcP interactions. Numerous kinetic studies[50,52–55,57,60–63] and computer simulations[5,58,59] suggest the presence of a high-affinity but low-reactivity binding site (the crystallographic one), and a low-affinity but high reactivity binding site that only functions at low ionic strengths. In this model the two Cc molecules do not interact with each other[12] and the low affinity binding site promotes the dissociation of Cc from the high affinity binding site (the only ET active site).[63–66] Various solution NMR studies (at an ionic strength of 120 mM, pH 6) describing the Cc:CcP complex as an highly dynamic one,[6,67–70] suggest a 1:1 model in which a single Cc molecule transiently binds CcP in different areas and orientations (see Chapter 1), one of which is the crystallographic structure.[5,59] It is assumed that under physiological conditions, the crystallographic complex is the active complex.[56] More details on the binding mode, ET and encounter complex between Cc and CcP are provided in the reviews [12] and [43] and in the following chapters.

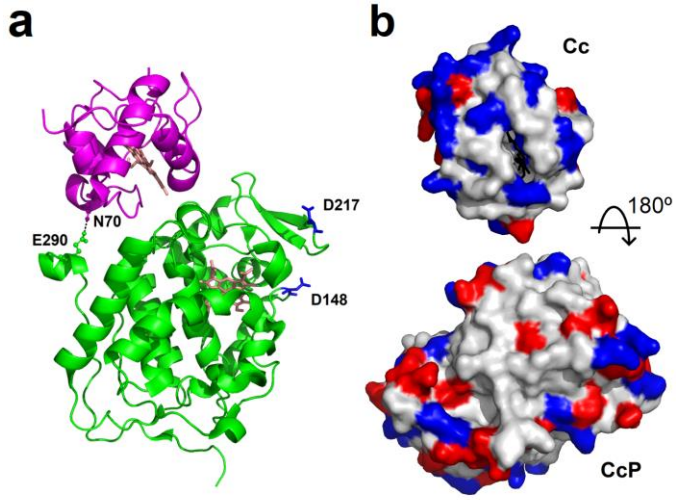

Figure 2.3 Cc:CcP interactions. a) Three-dimensional structure of the Cc:CcP complex: Cc in magenta ribbons and CcP in green ribbons. The haem groups in salmon sticks. The residues N70 and E290 (in spheres and sticks) stabilize the complex forming a single hydrogen bond (dotted line). The residues D217 and D148 (blue sticks) identify the secondary low-affinity binding site according to Van de Water *et.al.* 2015.[55] b) Distribution of charged residues in the crystallographic binding sites of Cc and CcP. The negatively charged residues are indicated in red and the positively charged ones in blue. (PDB entry 2PCC)[44]. The view shows the interfaces of the stereospecific binding site as present in the crystal structure.

Thesis outline

The aim of the research presented in this thesis is to test the importance of the charge distribution on protein surfaces for the formation protein complexes. The main question to be answered is whether optimization of the charge distribution is critical for rapid formation of an active complex. This question is addressed by introduction of new charged patches on the surface of CcP and studying the effects on complex formation with its partner Cc. It is predicted that disturbing the optimized charge distribution that guides Cc to the binding site for ET[2,5–7] by strong charged patches elsewhere will result in more futile encounters and thus a lower rate of active complex formation. The methods to test this hypothesis comprise Monte Carlo simulations of the electrostatic interactions during encounter complex formation, paramagnetic relaxation enhancement (PRE) experiments to probe the surface of CcP sampled by Cc and stopped flow experiments to measure association rates. **Chapter I** gives an introduction to the applications of paramagnetic NMR for the study of protein-protein interactions supported by several examples. **Chapter II** provides a brief introduction on the proteins used in this work, Cc and CcP. **Chapter III** describes the effect of the addition of a negatively charged patch, located on one side of CcP, on the encounter complex with Cc and the association rate of the protein complex. This approach is extended in **Chapter IV** to the kinetic characterization of five variants with different charge distributions on the CcP surface. **Chapter V** describes the effect of an additional negative patch on one side of CcP for complex formation with a variant of Cc that is known to spend more time in the encounter state with CcP.[71] **Chapter VI** provides a general discussion and concluding remarks. In combination, these studies show that additional charged patches can, in fact, enhance the formation of the active complex, resulting in additional productive encounters. On the other hand, upon disruption of the charged patch around the binding site, the additional charged patches lose their role in complex formation.

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