Review

The complex of cytochrome c and cytochrome c peroxidase: The end of the road?

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A R T I C L E   I N F O

Article history:
Received 23 June 2011
Received in revised form 21 July 2011
Accepted 22 July 2011
Available online 28 July 2011

Keywords:
Cytochrome c
Cytochrome c peroxidase
Protein complex
Electron transfer
NMR spectroscopy
X-ray crystallography

A B S T R A C T

Cytochrome c (Cc) and cytochrome c peroxidase (CcP) form a physiological complex in the inter-membrane space of yeast mitochondria, where CcP reduces hydrogen peroxide to water using the electrons provided by ferrous Cc. The Cc–CcP system has been a popular choice of study of interprotein biological electron transfer (ET) and in understanding dynamics within a protein–protein complex. In this review we have charted seven decades of research beginning with the discovery of CcP and leading to the latest functional and structural work, which has clarified the mechanism of the intermolecular ET, addressed the putative functional role of a low-affinity binding site, and identified lowly-populated intermediates on the energy landscape of complex formation. Despite the remarkable attention bestowed on this complex, a number of outstanding issues remain to be settled on the way to a complete understanding of Cc–CcP interaction.

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1. Introduction

Yeast cytochrome c peroxidase (CcP; ferrocytochrome-c : hydrogen-peroxide oxidoreductase, EC 1.11.1.5) was discovered in baker’s and brewer’s yeasts in 1939 [1]. Originally thought to be a soluble oxidase [1], CcP was soon realised to be a novel enzyme catalysing reduction of hydrogen peroxide and requiring reduced cytochrome c (Cc) for its activity [2]. Initial studies performed in the early 1960s established that CcP-catalysed conversion of H2O2 to water requires two reducing equivalents from ferrous Cc (Eq. (1)) and involves formation of Cc–CcP protein complex [3–6].

H2O2 + 2Cc2++ + 2H+ = 2Cc3++ + 2H2O

(1)

Since then, much effort has been devoted to elucidating the catalytic mechanism of this ostensibly simple reaction and understanding the process of intermolecular electron transfer (ET) from Cc to the active site of CcP. Despite enormous progress made over the past seven decades of research (Fig. 1), several questions concerning Cc–CcP complex formation and ET remain unanswered. Here we discuss literature pertaining to Cc–CcP interaction, including the papers published since 2002, the year of the latest comprehensive review on the subject [7]. For further information on individual proteins and a more detailed picture of earlier research on Cc–CcP system, the reader is referred to an excellent review by Vitello and Erman [7], a number of older works [8–10], and recent reviews on evolutionary [11] and structural [12] aspects of heme peroxidases.

1.1. Cytochrome c

Discovered in 1925 by Keilin [13], Cc is a key component of the eukaryotic respiratory chain, where it functions as an electron carrier between the membrane-bound Cc reductase and Cc oxidase. In yeast, Cc has other physiological partners, such as cytochrome b2 (also known as lactate dehydrogenase) and CcP [10]. The primary sequence of this protein, reported for more than 100 different species, is highly conserved among eukaryotes [10]. Two Cc isoforms, iso-1 and iso-2, are found in yeast, the former of which is much more widely studied and is referred to as yCc in this work. yCc is a positively-charged (pI = 9.54), low molecular weight (12.7 kDa) protein, consisting of 108 amino-acids and the heme prosthetic group. The native or recombinant protein can be readily expressed and purified from Saccharomyces cerevisiae [14–16] or Escherichia coli [17], respectively. Native yCc contains trimethylated lysine at position 72, a post-translational modification absent in the recombinant protein isolated from E. coli.

Cc is nearly spherical in shape and is formed by five α-helices and a short (β-strand (Fig. 2A), an overall fold that is highly conserved across the protein family [18]. Cc contains a c-type heme group that is located near the N-terminus and is attached to the polypeptide chain

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0005-2728/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2011.07.010
by covalent thioether bonds with two cysteine residues from the canonical CXXCH sequence (Fig. 2B). The heme contains a low-spin ($S = \frac{1}{2}$), six-coordinated iron that has two physiologically relevant oxidation states, Fe(II) and Fe(III), and is diamagnetic in the ferrous and paramagnetic in the ferric form. The coordination sphere consists of four pyrrole nitrogens of the heme, the N$_{eq}$ atom of H18, and the S$_{eq}$ atom of M80. To date, a number of high-resolution X-ray and solution NMR structures of cytochromes c from different species have been reported [10].

Owing to highly conserved primary sequences (Fig. 3) and very similar three-dimensional structures, several eukaryotic cytochromes – in particular horse Cc (hCc) – have been extensively used as structural and functional mimics of yCc. For many aspects of Cc–CcP chemistry, this has proven to be a valid approach; however, the physiological partner and functional mimics of yCc. For many aspects of Cc–CcP interaction

1.3. Cytochrome c peroxidase

Found in the intermembrane space of yeast mitochondria [19,20], CcP is a negatively-charged (pI 5.55), moderately-sized (34.2 kDa) protein consisting of 294 amino-acids and a non-covalently attached, b-type heme. Native or recombinant CcP can be isolated in a high yield from S. cerevisiae [21–23] or E. coli [24–27], respectively, and is the first heme enzyme for which a crystal structure was solved [28,29]. It is a highly α-helical molecule of cylindrical shape (Fig. 4A), with the heme group buried in a hydrophobic pocket within the protein and coordinated by the N$_{eq}$ atom of H175, the only axial ligand to the heme iron (Fig. 4B). The sixth coordination position, which remains vacant or is occupied by a water molecule in the resting state of the enzyme [30], is available for the binding of the peroxide substrate. In the resting state, CcP heme contains a high-spin ($S = \frac{7}{2}$), paramagnetic Fe(III) atom, which is oxidised to an Fe(IV)=O oxyferryl intermediate during the catalytic cycle (see below). In addition, a low-spin ($S = \frac{1}{2}$) form of Fe(II) with six-coordinate iron, approximating the ligation state of the enzymatic ferryl intermediate, can be prepared by cyanide binding to the vacant coordination position [21,31].

1.3. Cc–CcP interaction

In this section, we give a short overview of the key discoveries that have shaped Cc–CcP research over the past seven decades (Fig. 1) and outline the present state of affairs in this field. Early work on Cc–CcP complex, initially hampered by low yields and variable purity of CcP preparations, greatly benefited from the development of an efficient protocol that allowed isolation of a highly pure enzyme from S. cerevisiae [21]. Availability of sufficient amounts of both proteins enabled detailed kinetic and equilibrium studies, which demonstrated 1:1 Cc–CcP binding [32], proposed formation of 2:1 Cc–CcP complexes at low ionic strength [33], and refined the kinetic mechanism of peroxide reduction [5,6,34].

Now it is well established that the catalytic cycle consists of at least three steps (Eqs. (2)–(4)): reaction of CcP with peroxide to form Compound I (CpdI), an intermediate oxidised two equivalents above the native CcP(Fe$^{3+}$); one-electron reduction of CpdI by Cc(Fe$^{3+}$) to Compound II (CpdII); and subsequent reduction of CpdII by another Cc(Fe$^{2+}$) equivalent to regenerate the resting-state CcP(Fe$^{3+}$) enzyme [7].

\[ \text{CcP}(\text{Fe}^{3+}) + \text{H}_2\text{O}_2 = \text{CpdI} + \text{H}_2\text{O} \]  
(2)

\[ \text{CpdI} + \text{Cc}(\text{Fe}^{2+}) = \text{CpdII} + \text{Cc}(\text{Fe}^{3+}) \]  
(3)

\[ \text{CpdII} + \text{Cc}(\text{Fe}^{2+}) = \text{CcP}(\text{Fe}^{3+}) + \text{Cc}(\text{Fe}^{3+}) \]  
(4)

In CpdI the heme iron is oxidised to Fe(IV)=O oxyferryl group [35,36] and the side chain of W191 is oxidised to a cationic indole radical [W191$^{•+}$] [37–39]. CpdI, a one-electron reduction product of CpdII, contains either an Fe(IV)=O (CpdII$^{•+}$) or W191$^{•+}$ (CpdII$^{2•}$) species, depending on experimental conditions [40,41]. The last two
reactions (Eqs. (3) and (4)) involve complex formation with and ET from ferrous Cc. Since the pioneering studies in the early 1970s, most of the research in Cc–CcP field has been focused on understanding the process of protein complex formation and unravelling the mechanistic details of the two ET steps.

As shown by a variety of experimental techniques, and confirmed by the crystal structure of Cc–CcP complex [42], the interaction between Cc and CcP occurs with a 1:1 stoichiometry in a broad range of experimental conditions. However, several reports demonstrated formation of a 2:1 complex at low-ionic strength, with the binding of the second Cc molecule 2–4 orders of magnitude weaker than that of the first [43–46]. Since then, location of the second Cc binding site on the surface of CcP has been a matter of debate that has given rise to two contrasting views on the Cc–CcP interaction. The first concept postulates a unique 1:1 protein complex with the high-affinity Cc binding site as seen in the crystal structure [42] and a second, much weaker binding site in the 2:1 complex, with no interactions between bound Cc molecules. Another hypothesis proposes multiple forms of the 1:1 complex, with Cc bound to different locations on the surface of CcP, and suggests that the protein–protein orientation seen in the crystal structure represents only a sub-population of binding geometries [9,47,48]. In this view, formation of a 2:1 complex is inhibited by strong electrostatic repulsion between bound Cc molecules.

The two binding models have engendered two different mechanisms for the intermolecular ET between Cc and CcP. According to one view – termed here “one-site ET” and propounded primarily by Durham, Millett and co-workers (Section 4.2) – the ET occurs only from the Cc bound to the high-affinity site as seen in the X-ray structure. This model assumes interaction between low- and high-affinity sites at low ionic strength, with Cc binding to the former promoting Cc dissociation from the latter. An alternative mechanism – dubbed here “two-sites ET” and proposed by Hoffman and colleagues (Section 4.3) – posits multiple forms of the ET active 1:1 complex and postulates that the ET from the second, weakly bound Cc is faster than that from the Cc bound to the high-affinity site. In what follows, we review experimental evidence for the two binding models and the ensuing ET mechanisms and discuss recent developments in the study of Cc–CcP complex.

2. Cc–CcP complex formation

2.1. Binary complex

The first direct evidence for 1:1 Cc–CcP complex formation, suggested by earlier kinetics studies [3–6], came from the work of Nicholls and Mochan [32,34,49], who showed by analytical ultracentrifugation and size-exclusion chromatography that Cc binds to the resting-state CcP and its CcP* derivative (CcPCN) with equilibrium dissociation constant ($K_D$) of 20 μM in 55 mM sodium phosphate pH 7.0 at 25 °C [32]. The elution profile of Cc–CcP complex on a gel-filtration column revealed the presence of free and bound proteins, indicative of an association–dissociation equilibrium typical of a reversible complex formation [49]. Further studies by a variety of equilibrium techniques, including spectrophotometry [50], analytical ultracentrifugation [51], fluorescence quenching [52,53], affinity chromatography [54], potentiometry [46], nuclear magnetic resonance (NMR) spectroscopy [55–59], and isothermal titration calorimetry (ITC) [60–66], have confirmed formation of a 1:1 complex of CcP with both yeast and horse Cc. At pH 6.0 and 25 °C, the measured dissociation constants of yCc–CcP and hCc–CcP complexes are in the range of 0.01–72 μM (1 = 18–200 mM) and 0.23–500 μM (1 = 3.5–200 mM), respectively (see Tables 2 and 3 in ref. [7]).

Several general conclusions can be made from these studies. First, binding of both yCc and hCc to CcP becomes weaker with increasing ionic strength, confirming the importance of electrostatic forces in Cc–CcP complex formation. Second, yCc binding to CcP is stronger than that of hCc in the entire range of experimental conditions, suggesting a clear binding preference for the physiological partner. This preference and a higher turnover observed with yCc than any other cytochrome [33] indicate a co-evolution of yCc and CcP, as was suggested for eukaryotic Cc and Cc oxidase [67]. Third, a number of studies show that, for both yeast and horse Cc, binding to CcP is virtually independent of Cc reduction state [32,46,53,59,66], consistent with Cc(Fe$^{3+}$) product inhibition observed by kinetics [3,34]. This finding runs contrary to the conclusion of Hake et al. [54], who reported a 50-fold stronger binding of CcP to ferrous than ferric Cc immobilized on a Sephadex column. As mentioned before [7,46], this drastic difference in binding is most likely due to column matrix
effects. Finally, it appears that Cc–CcP interaction is independent of the CcP spin-state [32,55,57,68], with both yeast and horse Cc binding equally well to a high-spin, resting state CcP or a low-spin CcP derivative [32,57]. Given that CcP contains is considered a good mimic of Fe(IV) = O oxyferryl species in CpdI and CpdII intermediates [57,69], this seems to suggest that, as far as “one-site” ET model is concerned, both ET steps (Eqs. (3) and (4)) take place in Cc-CcP complexes of the same binding affinity.

2.2. Ternary complex

Formation of a ternary Cc–CcP complex was first proposed in 1977 by Margoliash and co-workers to explain biphasic steady-state kinetics curves and gel-filtration profiles observed for the interaction of CcP with horse and iso-2-yeast Cc [33]. However, soon afterwards it was shown that the kinetics data could be explained equally well by an independent, stepwise binding of two Cc molecules to a single, catalytically-active site on CcP [70], and it was argued that the accompanying size-exclusion experiments overestimated the amount of bound Cc [7,50]. A related study of CcP interaction with iron-free porphyrin derivative of hCc by fluorescence quenching and analytical gel-filtration also suggested the presence of higher-order complexes at low ionic strength [71]. Yet it too suffered from a number of experimental drawbacks such as likely matrix effects on a Sephadex column (run at I = 4.2 mM) and a possible nonspecific binding of CcP by the surface-active porphyrin Cc.

The first conclusive evidence for 2:1 Cc–CcP complex formation came from studies of Hoffman and co-workers [43–45], who followed triplet-state quenching of Zn-substituted CcP or Cc by varying concentrations of the respective hCc partner in the Fe(III) state. The observed, rather complex, kinetics are well explained by a model postulating two non-interacting Cc binding sites of markedly different affinities and reactivities, with faster ET from Cc bound at the low-affinity site (see Section 4.3.). Shortly afterwards, Mauk et al. [46] directly determined formation of a 2:1 yCc-CcP complex using potentiometry, a non-kineti, equilibrium technique. The proton titration curves fit well to 1:1 and 2:1 binding models at I ≥ 100 mM and I ≤ 50 mM, respectively, with ca. 1,000-fold difference in the binding constants for the low- and high-affinity sites at I = 50 mM pH 6.0 25 °C. Interestingly, Cc binding to the high-affinity site is accompanied by proton release, while Cc interaction at the low-affinity site triggers proton uptake, suggesting different electrostatic properties of the two binding domains [46].

An ITC study by Leesch et al. [63], carried out in 10 mM phosphate at pH 7.0 and 25 °C, has provided another indication for the presence of a ternary hCc–CcP complex. However, the heat changes attributed to the binding of the second Cc molecule were very small, so that no reliable thermodynamic parameters for the binding to the low-affinity site could be obtained. From this limited experimental dataset, the authors estimated K_d ≥ 0.2 mM for the low-affinity site and suggested a negative binding cooperativity between two Cc molecules, with an estimated ΔG penalty of 0.8 kcal/mol for the second binding event [63]. Finally, another ITC study has demonstrated that the tri-saccharides melizitose and raffinose promoted formation of a 2:1 complex between yCc and CcP at I = 50 mM pH 6.0 25 °C [72]. Given that addition of mono-, di-, and tetrasaccharides has virtually no effect on Cc–CcP binding [65], the observed stabilisation of the ternary complex is most likely due to specific interactions of the studied trisaccharides with Cc and/or CcP, rather than crowding effects suggested by the authors.

2.3. Localising Cc–CcP binding domains

2.3.1. Chemical modification and early cross-linking studies

Early on it was established that electrostatics play a predominant role in Cc–CcP interaction [3,49,73], as would be expected from oppositely-charged molecules. In particular, it was shown that the reaction is inhibited by polycations such as salmine [3] and polylysine [73], both of which act as competitive inhibitors of Cc binding. In addition, it was observed that polycetylation, but not polyguanidination, of Cc lysines impaired its binding to CcP and abolished the catalytic activity, highlighting the importance of the positive charge on Cc, rather than Lys residues as such [73]. These findings rationalised the observed ionic strength dependence of catalytic peroxide reduction by the Cc–CcP pair [6,34]. More recent work has illustrated that Cc–CcP binding can also be abolished by small polylysine peptides [74] and functionaised charged nanoparticles [75].

Complex formation between Cc and CcP was shown to inhibit ascorbate reduction [76] of and cyanide binding [77] to Cc(Fe³⁺) and have no effect on the binding of hydrogen peroxide and fluoride to CcP [77]. These results suggested that the exposed heme edge of Cc is buried in the interface of the protein complex and Cc binding does not occlude the channel leading to the active site of CcP. This agreed with the previous NMR work of Gupta and Yonetani [55], and later Erman and co-workers [56–58], who showed that the heme methyls of Cc exhibit large spectral changes upon binding to CcP. Using an ingeniously simple experiment, Gupta and Yonetani [55] estimated the distance between the heme groups in hCC-cCcP complex. The authors reasoned that, if the two groups were close, changes in the spin-state of CcP heme iron – effected by addition of CN⁻ or F⁻ ligands to the resting-state enzyme and leading to drastic changes in paramagnetic properties of Fe³⁺ atom – would affect NMR resonances of hCc heme methyls. As no such effects were observed, the authors concluded that the two heme groups must be separated by at least 25 Å [55].

In a pioneering chemical modification approach, Margoliash and co-workers used hCc modified with carboxydinitrophenyl and trinitrophenyl groups at specific lysine residues as a probe of the interaction with CcP [78]. They found that modification of residues 13, 27, 72, 86, and 87 resulted in large changes in the observed kinetics parameters, while alteration of lysines 8, 25, and 73 had little effect. The authors concluded that the former group of residues, located at the front face of hCc surrounding the exposed heme edge, defines the CcP binding interface, while the latter three lysines sit at the periphery. In addition to mapping out the hCc region involved in CcP binding, this study established that “… the net charge of a particular region and not of the entire molecule, is the major parameter [in the interaction of Cc with CcP]” [78]. The main conclusions of this work were confirmed in a related study by Smith and Millett [79], who showed that trifluoroacetylation of hCc lysines 13, 25, 79, and 87 decreased the reaction rate with CcP, while that of 22, 55, 88, and 99 did not.

Recognising the importance of electrostatic interactions in the protein complex formation, and inspired by a similar approach used for the redox pair of Cc and cytochrome b₅ [80], Poulos and Kraut constructed a model of Cc–CcP complex by visual optimisation of the complementary charges on the two proteins [81]. Starting from available X-ray structures of tuna Cc [82] and yeast CcP [29], the authors matched a patch of aspartate residues on CcP with a ring of lysines surrounding the exposed heme edge of Cc. The resulting model exhibited five complementary charge interactions and two additional hydrogen bonds across the interface (K13, K27, K72, K86, K87, Q12, Q16 of Cc with D37, D79, D216, D37, D34, N87, and Q86 of CcP, respectively), a remarkable steric fit of the two proteins, and an unexpected coplanarity of the two heme groups [81]. The Cc–CcP binding model agreed well with the results of the earlier chemical modification study [78], and the separation between heme groups was found to be in the range of the experimentally-determined heme-

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3 yCc and CcP charges are, respectively, +6 and −4 at pH 6.0 as estimated from protein titration curves [206,207].
to-heme distances [52,55]. This seminal work stimulated numerous experimental studies aiming to validate the hypothetic model and assess the role of the proposed interacting residues in the complex formation.

Further chemical modification work confirmed the importance of hCc residue K13 [83] and indicated a possible involvement of CcP residue H181 [84] in the interaction with the binding partner. Modification of CcP carboxylate groups with carbodiimide reagent was found to inhibit peroxidase activity, while acetylation of CcP lysines showed little effect, once again demonstrating the significance of the negative charge on CcP [85]. When the reaction with carbodiimide was carried out in the presence of hCc, a number of CcP glutamates and aspartates became protected [86]. These residues mapped out to two regions on the CcP surface: a negatively-charged patch at the front face of the molecule (harbouring D33, D34, E35, D37, E290, E291 and the C-terminal carboxylate of L294) and a pair of E221 and D224, located towards the bottom (Fig. 5). The authors noted that “Since cytochrome c is a globular protein of considerably smaller size than cytochrome peroxidase it is impossible for a single molecule of cytochrome c to shield at once all of the above carboxyl groups.” [86] An important conclusion from this work is that the interaction between Cc and CcP appears to involve a broader binding surface, rather than a single binding geometry implied in Poulos and Kraut’s model.

In an approach complementary to the chemical modification strategies discussed above, several groups attempted to “freeze-in” the reactive Cc–CcP complex by chemical cross-linking [83,85–87,89]. Thus, Bisson and Capaldi [89] prepared hCc derivatives modified with a photoactivatable arylazido group at specific lysine residues. Upon addition of the binding partner and subsequent photo-activation, hCc residue K13 became cross-linked to CcP residue(s) located in 50 amino acid-long N-terminal stretch as established by chemical cleavage peptide mapping. The resulting cross-link exhibited no peroxidase activity towards exogenously added Cc, indicating that functional Cc binding site was blocked. Similar experiments with hCc modified at K22, located at the back of the protein, yielded no intermolecular products, confirming the specificity of the Cc–CcP interaction [83]. Using a water-soluble carbodiimide derivative – EDC, a “zero-length” cross-linker promoting amide bond formation between amino and carboxyl sidechains – Bosshard and co-workers prepared covalent hCc–CcP complexes of 1:1 stoichiometry [85,87,89]. Peptide mapping of the purified product showed the presence of two major cross-links, those of hCc K13 or K86 linked to CcP acidic residues in 32–37 region. Using a similar, slightly modified, cross-linking procedure [90], Erman and co-workers isolated a single, covalent hCc–CcP product and showed that NMR spectral changes exhibited by heme groups of both Cc and CcP in the cross-link are identical to those observed in the native, non-covalent complex, confirming the validity of the cross-linking approach [90,91]. Many early cross-linking studies reported certain heterogeneity of the products [85,87–89], corroborated by subsequent kinetics [92] and NMR [91,93] analyses, suggesting that more than one binding geometry might be sampled in the course of Cc–CcP interaction. Interestingly, comparison of the cross-linked products of CcP with hCc and yCc by NMR revealed larger structural heterogeneity in the latter [91,93], once again demonstrating clear species-specific differences in Cc–CcP interaction (see Section 5). In addition, higher yields of yCc–CcP cross-links at high rather than low ionic strength and exactly the opposite observation for hCc–CcP covalent products were interpreted as evidence for a larger impact of intermolecular electrostatic forces in hCc–CcP complex formation [91].

2.3.2. X-ray crystallography

Suggested back in 1971 by Nicholls and Mochan [32], co-crystallisation and X-ray structure determination of Cc–CcP complex were at first unsuccessful [94]. However, several years after, Pelletier and Kraut [42] succeeded in solving crystal structures of both yCc–CcP and hCc–CcP 1:1 complexes (Fig. 6). Contrary to an earlier electrostatic model [81] and Brownian dynamics simulations [47,48] that predicted numerous strong interactions across the interface, only two intermolecular salt bridges and a hydrogen bond (K8-N38, K72-E290, and K87-E35; Cc residues given first) were observed in hCc–CcP structure (Fig. 6A). Remarkably, a sole, weak intermolecular hydrogen bond (N70–E290, Cc residue given first) mediates protein–protein interaction in yCc–CcP complex (Fig. 6B). Both complexes appear to be stabilised mainly by overall electrostatic potential of the protein molecules and van der Waals interactions [42]. Noticing that the heme group of yCc is in van der Waals contact with the CcP surface, Pelletier and Kraut proposed an ET pathway, which would require a single, through-space electron jump from Cc heme to A194 or A193 residue of CcP, followed by a through-bond travel via an A193-G192-W191 conduit (Fig. 6D). In contrast, the closest distance between hCc heme and CcP is 7 Å, rendering direct ET from the crystallographic orientation inefficient (Fig. 6C). The authors suggested that weakening of the intermolecular charge–charge interactions (e.g. at higher ionic strength), followed by subtle re-orientation of hCc molecule could bring it into a favourable ET arrangement, similar to that of yCc–CcP [42]. Heme-to-heme distances in the two crystallographic complexes are compared to those determined in earlier solution studies in Table 1.

To solve hCc–CcP structure, Pelletier and Kraut partially dehydrated protein crystals before data collection, an idiosyncrasy found to improve X-ray diffraction [42] but raising concerns that the accompanying changes in protein hydration might force the molecules into a “non-native” orientation [9]. This contentious issue has been settled by Crane and co-workers [96], who solved the X-ray structure of hCc complex with Zn-substituted CcP (ZnCcP) using ‘wet’ crystals, showing that the two structures are virtually identical. To date, a number of Cc–CcP structures have been reported (Table 2); all of them exhibit small, poorly packed, rather hydrophilic binding interfaces, typical of transient complexes of redox proteins [103]. In order to assess whether the crystallographic Cc–CcP orientation is representative of the binding geometry in solution, Crane and co-workers [96] compared triplet state quenching of ZnCcP by Cc in crystal and solution (see Section 4.3). The authors found a remarkably close match between the quenching constants, concluding that crystal and solution structures must be the same. This finding was independently confirmed by an NMR study, which showed that crystallographic yCc–CcP orientation is indeed the dominant form of the complex in solution [100].

In retrospect, Poulos and Kraut’s model [81] appears over-conservative: snug steric fit of two proteins and seven hydrogen bonds across the interface would greatly reduce the dissociation rate constant (koff), effectively limiting enzyme turnover. Comparison of this hypothetical model with the X-ray structures of the complex
Experimentally-determined heme-to-heme distances in Cc of the Cc work of Pelletier and Kraut dissociation, ultimately optimising for efficiency, while the latter balanced the need for high affinity and fast dissociation, ultimately optimising for efficiency. The ground breaking work of Pelletier and Kraut [42] paved the way for subsequent studies of the Cc–CcP structure–function relationship by the combination of site-directed mutagenesis and biophysical techniques.

2.3.3. Site-directed mutagenesis

Since the preparation of the first, site-specific Cc mutants in S. cerevisiae [22] and E. coli [104], a number of variant Cc–CcP complexes have been investigated. Corin and co-workers [54,105,106] studied the effects of charge-reversal D37K, D79K, and D217K CcP mutations on the interaction with yeast and horse Cc by a combination of kinetic and equilibrium techniques. They showed that substitution of CcP D37 residue, sitting close to the crystallographic binding site, leads to destabilisation of Cc–CcP complexes, while mutation of the latter two aspartates – located further away on CcP surface – have little effect. In a series of works, Millett and co-workers [107–110] studied the interaction of horse and yeast Cc with E32Q, D34N, E35Q, E290N, E291Q, and A193F CcP variants, designed specifically to probe the crystallographic binding domain. Using stopped-flow kinetics and photo-induced ET measurements, the authors demonstrated that CcP residues D34, E35, A193, and E290 (but not E32 and E291) are involved in Cc–CcP complex formation, in good agreement with X-ray work [42]. These findings were confirmed in an ITC study of Erman et al. [61], who detected a 3–4 fold decrease in hCc affinity for D34N, E32Q, E35Q, A193F, and E290N CcP (all residues located within Cc binding site), but not its E291Q variant (sitting at the periphery, with the sidechain pointing away from the bound Cc). The observed effects and the accompanying changes in thermodynamic parameters agreed well with the X-ray structure of hCc–CcP complex [42].

Pielak and Wang [64] also used ITC to investigate the binding of the wild-type (wt), A81G, K72A, and K87A yCc to CcP D34A, V197A, and E290A mutants to probe the high-affinity, crystallographic site; and wt yCc interaction with D148A, D217A, and L182A CcP to explore secondary Cc binding regions. The authors found: 1) a modest, 1.7 to 4-fold decrease in CcP binding affinity for K72A and K87A yCc variants; 2) little influence of CcP mutations designed to interrogate non-crystallographic binding sites; 3) large (∆ΔG > 2 kcal/mol) effects of V197A and E290A CcP substitutions4; and 4) surprising, stabilising effect of D34A CcP mutation, leading to a 4-fold increase in the binding constant. Given the central location of D34 residue in the yCc-CcP interface as seen in X-ray structure [42] and considering the destabilising effect of D34N mutation in hCc–CcP complex [61], the underlying structural causes of enhanced D34A CcP–yCc binding remain unclear [7]. Furthermore, using double-mutant cycles, Pielak and Wang discovered a stabilising interaction between Cc A81 and CcP V197 sidechains (coupling energy ∆ΔGc = −1.9 ± 0.7 kcal-mol−1) [64], in excellent agreement with X-ray structure showing multiple van der Waals contacts between the two residues [42].

In an impressive experimental effort, Erman and co-workers [111,112] prepared 46 charge-reversal CcP variants, mutating each of the 20 glutamates and 24 out of 25 aspartates to lysine residues and introducing two positive-to-negative R31E and K149D substitutions, introduced to probe the crystallographic binding domain. Using Y197 sidechains (coupling energy ∆ΔGc = −1.9 ± 0.7 kcal-mol−1) [64], in excellent agreement with X-ray structure showing multiple van der Waals contacts between the two residues [42].

Table 1

<table>
<thead>
<tr>
<th>Distance, Å</th>
<th>Complex</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;25</td>
<td>hCc–CcP</td>
<td>NMR [55]</td>
</tr>
<tr>
<td>14.9 ± 0.5</td>
<td>yCc–CcP</td>
<td>FRET [52]</td>
</tr>
<tr>
<td>7 (17.5 ± 2.5)</td>
<td>hCc–CcP</td>
<td>Excitation spectroscopy [95]</td>
</tr>
<tr>
<td>23.5 ± 0.5</td>
<td>yCc–CcP</td>
<td>FRET [71]</td>
</tr>
<tr>
<td>19.1 (26.5)</td>
<td>yCc–CcP</td>
<td>X-ray [42]</td>
</tr>
<tr>
<td>22.4 (30.0)</td>
<td>hCc–CcP</td>
<td>X-ray [42]</td>
</tr>
</tbody>
</table>

4 The reported value of ∆ΔGc = −1.9 ± 0.2 kcal/mol, translating into ca. 35,000-fold decrease in the binding affinity relative to the wt complex, is most likely erroneous; see [7] for details.
one at a time. Examining their interaction with yCc by steady-state kinetics, the authors found that only 5 out of 46 CcP mutants (R31E, D34K, D37K, E118K, and E290K) exhibited reduced af

Summary of the Cc–CcP structures deposited in the Protein Data Bank at the time of writing. The interface analysis was performed with ProtodP [102]. For structures containing two complexes in the asymmetric unit, the averages of the values calculated for both individual complexes are given.

<table>
<thead>
<tr>
<th>PDB entry</th>
<th>Complex</th>
<th>Resolution (Å)</th>
<th>RMSD (Å)</th>
<th>Interface size (Å²)</th>
<th>% Buried surface</th>
<th>Interface polarity</th>
<th>Gap index (Å)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>2PCC</td>
<td>yCc–CcP</td>
<td>2.3</td>
<td>0.63³</td>
<td>1107</td>
<td>47/46</td>
<td>37/47</td>
<td>4.9</td>
<td>[42]</td>
</tr>
<tr>
<td>2PDC</td>
<td>hCc–CcP</td>
<td>2.8</td>
<td>6.60²</td>
<td>1030</td>
<td>50/49</td>
<td>61/58</td>
<td>5.3</td>
<td>[42]</td>
</tr>
<tr>
<td>1U74</td>
<td>yCc–ZnCcP</td>
<td>2.4</td>
<td>0.58</td>
<td>1170</td>
<td>54/53</td>
<td>39/45</td>
<td>4.8</td>
<td>[96]</td>
</tr>
<tr>
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<td>49/47</td>
<td>59/55</td>
<td>4.1</td>
<td>[96]</td>
</tr>
<tr>
<td>156V</td>
<td>A81C yCc–Y197C CcP covalent crosslink</td>
<td>1.88</td>
<td>2.82</td>
<td>569</td>
<td>26/25</td>
<td>21/34</td>
<td>11.5</td>
<td>[97]</td>
</tr>
<tr>
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<td>F82S yCc–ZnCcP</td>
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<td>0.56</td>
<td>1292</td>
<td>56/56</td>
<td>40/49</td>
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<td>[98]</td>
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<tr>
<td>2B11</td>
<td>F82W yCc–ZnCcP</td>
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<td>0.52</td>
<td>1150</td>
<td>52/52</td>
<td>40/46</td>
<td>4.8</td>
<td>[98]</td>
</tr>
<tr>
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<td>19.02</td>
<td>757</td>
<td>32/33</td>
<td>43/30</td>
<td>7.7</td>
<td>[98]</td>
</tr>
<tr>
<td>2BOZ</td>
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<td>2.7</td>
<td>18.54</td>
<td>825</td>
<td>37/41</td>
<td>44/40</td>
<td>5.6</td>
<td>[98]</td>
</tr>
<tr>
<td>2BCN</td>
<td>yCc–ZnCcP⁰</td>
<td>1.7</td>
<td>0.75</td>
<td>1185</td>
<td>51/48</td>
<td>44/42</td>
<td>5.1</td>
<td>[99]</td>
</tr>
<tr>
<td>2GB8</td>
<td>yCc–CcP</td>
<td>N/A¹</td>
<td>2.02</td>
<td>1320</td>
<td>58/52</td>
<td>35/46</td>
<td>4.5</td>
<td>[100]</td>
</tr>
<tr>
<td>2P1</td>
<td>T12A yCc–CcP</td>
<td>N/A¹</td>
<td>1.50</td>
<td>1445</td>
<td>65/68</td>
<td>39/48</td>
<td>3.6</td>
<td>[101]</td>
</tr>
</tbody>
</table>

¹ Root mean square deviation calculated for Cc Cα atoms after superimposition of CcP molecules in the query and the corresponding yCc–CcP or hCc–CcP Pelletier and Kraut structure.

² Interface size, defined as the sum of accessible surface areas (ASA) buried by two proteins upon complex formation.

³ Percent of ASA buried by each protein in the complex; Cc values are given first.

⁴ The percentage of the interface area composed of polar atoms; Cc values are given first.

⁵ Gap index is defined as the gap volume, a volume of the empty space between interfacial atoms, divided by the interface size.

⁶ Calculated between two complexes in the asymmetric unit.

⁷ Calculated with 2PCC structure as a reference.

⁸ The asymmetric unit of this structure, solved in D₀₂, contains 1 Cc and 2 CcP molecules, forming two intermolecular interfaces.

⁹ Backbone-resolution structures solved by solution NMR. All values are calculated for the first model in the ensemble.

3. Dynamics in Cc–CcP interaction

It is generally agreed that, except for sidechain reorganisation at the binding interface, Cc–CcP complex formation does not lead to conformational changes of individual proteins as evidenced by analytical ultracentrifugation [49], X-ray crystallography [42,96], and NMR spectroscopy [59]. However, small binding-induced perturbations of the CcP heme environment were observed by high-resolution fluorescence [116], UV-visible [74], NMR [57,69], and resonance Raman [117] spectroscopies. As the CcP heme group is buried inside the protein core, these findings indicate a certain conformational flexibility of the CcP molecule and suggest a possibility that Cc binding event is propagated to the enzyme’s active site [69]. Still, the observed effects are very small, so that for most practical purposes interacting Cc and CcP can be treated as rigid-bodies. In this context, the dynamics observed in Cc–CcP complex and discussed below arise from variations in relative orientations of protein molecules rather than binding-induced structural changes of individual proteins.

3.1. Molecular modelling

Early availability of crystal structures of individual proteins and the general agreement on the dominant role of electrostatics in Cc–CcP interaction encouraged theoretical modelling of Cc–CcP complex. Early on it was established that an asymmetric charge distribution on the CcP interaction encouraged theoretical modelling of Cc and known to control the intermolecular ET as seen by X-ray crystallography [113] and confirmed a central role of F82 [98], mediating Cc–CcP interaction as seen by X-ray [42] and NMR [59,93] and known to control the intermolecular ET [114,115]. Another work demonstrated that alanine substitution of yCc residues R13 and T12, two more sidechains located in the Cc–CcP interface, led to a 30-fold decrease and a 10-fold increase in CcP binding affinity, respectively [66]. Just as in the case of the D34A CcP mutation discussed above [64], structural determinants of the tighter binding in T12A Cc–CcP complex are not known. Using double-mutant cycles, Volkov et al. [66] showed that Cc R13 interacts with Y39 residue of CcP, in agreement with the crystal structure of the complex [42] exhibiting van der Waals contacts between the two sidechains. Given their central location in Cc–CcP interface and large effects of their alanine substitutions on the binding energetics, the authors proposed that Cc R13 and CcP Y39 are part of a binding ‘hot spot’ [66].

2.3.4. Localising low-affinity binding site

Experimental localisation of a secondary Cc binding site(s) on the surface of CcP has been exceedingly hard due to a large difference in the binding constants for the low- and high-affinity sites (10³–10⁵-fold, see Section 2.2), complicating detection of the former in the presence of the latter. So far, only two studies have risen to this difficult task. Both of them used mutagenesis to assess the role of CcP residues located in a negatively-charged region identified as a secondary Cc binding site in Brownian dynamics simulations (discussed in detail in Section 3.1) [47,48]. Using ITC and triplet state quenching of ZnPc by hCc, Leesch et al. [63] showed that the charge-reversal K149E CcP mutation leads to a 10-fold increase in Cc binding to the low-affinity site, while D148K substitution has little effect. Given the proximity of the two residues and the central location of D148 in the putative binding site, the latter finding is difficult to rationalise. In contrast, in their ITC study of a 2 : 1 yCc–CcP complex stabilised by trisaccharide melezitose, Morar and Pielak [72] conclusively demonstrated that D148A mutation of CcP abolishes ternary complex formation. Taken together, these results suggest that CcP residues D148 and K149 define the low-affinity Cc binding site.
simulations of Northrup et al. [121] demonstrated the importance of dipole-induced electrostatic torques in accelerating Cc–CcP association to the experimentally observed values.

Extending these ideas further, Northrup and co-workers [47,48] performed detailed BD simulations of hCc–CcP complex employing atomic-resolution protein structures, the Poisson–Boltzmann equation to calculate electrostatic interaction potentials, and realistic reaction criteria based on heme-to-heme distances (d) and angles (\(\phi\)). The analysis of the BD trajectories – leading from fully separated protein molecules to formation of Cc–CcP encounters and subsequent dissociation or conversion to a reactive protein complex – provided information on possible binding geometries and probabilities of the productive encounters, which could be further converted into the association rates (\(k_{on}\)). Several important conclusions came from this work. First, instead of converging to a single, unique solution, the simulations produced a large ensemble of electrostatically favourable encounter complexes, predominantly localised in three CcP regions: Site 1, clustered around D34 and containing the crystallographic high-affinity Cc binding site; Site 2, centred on D148 and comprising ET-competent docking geometries at the back of the protein; and Site 3, or “D217 face” [47], connecting the latter two regions (Fig. 7A). Based on this observation, the authors concluded that “... there is no dominant complex which forms, but a surprisingly even distribution over a variety of complexes” and “... there is not [sic] strict charge complementarity in operation that locks the proteins into a single electron transfer arrangement, but that association is more non-specific in nature” [48]. Second, the analysis revealed that the electrostatic stabilisation of Cc–CcP complexes was achieved by 2 to 4 intermolecular ionic contacts, confirming the importance of certain charged patches rather than the total protein charges. Third, the authors noticed that “… cyt c undergoes numerous rotational reorientations … as it explores an extensive region on the surface of cyt c per[oxidase], and may even include all three major docking regions in one excursion” [48]. This finding suggested a possible communication between spatially separated CcP Sites 1 and 2, with region 3 acting as an intervening conduit. Finally, analysis of the simulated association rates revealed that a combination of the contributions from both Sites 1 and 2 is required to reproduce experimental \(k_{on}\) values, suggesting a “… necessity for electron transfer to occur from a range of electrostatically favourable encounter geometries” [48]. Intriguingly, the best match with the experimental \(k_{on}\) was found for docking solutions with \(d\leq 20\) Å and \(\phi\leq 60^\circ\), the binding geometry observed later in the X-ray structure [42]. The general conclusions of Northrup et al. [47,48] were confirmed in a subsequent modelling study by Warwicker [122], whose electrostatic energy maps revealed extensive hCc–CcP interaction surface, with Cc molecule experiencing considerable rotational motion, again suggesting a flexible binding interface, rather than a single, static Cc–CcP complex.

BD simulations of yCc–CcP system [123,124] employed different reaction criteria, namely intermolecular polar contacts in the crystallographic complex, which provide accurate association rates for the dominant binding geometry, but give no information on the overall distribution of protein encounters as observed for hCc–CcP. The simulated \(k_{on}\) constants are in a good agreement with the experimental values [123,124], suggesting that most of the ET can occur from the high-affinity Cc–CcP site as seen in the X-ray structure [123]. Even with such restrictive reaction criteria, Gabdouline and Wade [124] observed an extended distribution of yCc–CcP encounters in the area surrounding the crystallographic binding site and reported considerable rotational motion of the Cc molecule, similar to what was seen for hCc–CcP system (see above). Recent Monte Carlo (MC) simulations used atomic partial charges to calculate Poisson–Boltzmann electrostatic potentials and a uniform distance cut-off as a sole reaction criterion to generate an ensemble of yCc–CcP encounters [125]. Overall, the encounter distribution is very similar to that obtained for hCc–CcP complex by Northrup et al. [47,48] except for a shallower D148 region (Fig. 7B), which can either be due to differences in the simulation protocols or reflect species-specific variation in Cc charge distribution.

### 3.2. Experimental evidence

The first experimental evidence for dynamics in Cc–CcP complex came from early chemical modification and cross-linking studies (Section 2.3.1.), which suggested that multiple binding geometries might be sampled in the course of Cc–CcP interaction. This conclusion was indirectly confirmed by crystallographic study of Poulos et al. [94], who showed that electron density maps of hCc–CcP co-crystals contain only CcP molecules, while hCc is orientationally disordered and occupies the space between CcP dimers. A possible explanation of the observed results proposed that the interaction between the two proteins occurs over a broad binding surface, leading to dynamic disorder of hCc molecules.

In their studies of time-resolved fluorescence resonance energy transfer (FRET) between Mg-substituted CcP (MgCcP, donor) and Cc (acceptor), McLendon and co-workers [126,127] found a striking difference between FRET decay curves obtained at 300 K and 77 K. While the former showed a simple, monoexponential behaviour, the latter exhibited far greater complexity and required at least three
separate components for a reliable fit. The complex lineshape indicates a distribution of decay times arising from a distribution of donor-acceptor distances, which implies the presence of multiple protein–protein orientations, each of which will have a characteristic donor-acceptor distance and angle and, thus, a characteristic energy transfer rate [126]. Analysis of the decay curves provided a distribution of donor-acceptor separations in yCc–CcP system (Fig. 8), which agrees well with heme-to-heme distances determined in other studies (Table 1). As noted by the authors, at 77 K “the distribution of protein complexes would be frozen out and could not rearrange on the time scale of fluorescence (10−8 s), while at 300 K “small amplitude motions at the interface would allow these conformers to equilibrate. If equilibration occurred on nanosecond time scale, the distributed kinetics found at low temperatures would be largely or completely washed out.” [127] Thus, this work presented a picture of a dynamic protein complex consisting of multiple, rapidly interconverting protein–protein orientations of similar binding energies, which became a basis of McLendon’s “Velcro” model [128] for the interaction between redox proteins.

Subsequent studies of hCc–CcP [129] and yCc–CcP [130] interactions by hydrogen-deuterium exchange NMR spectroscopy revealed large protection factors for the residues localized on the front face of Cc, consistent with complexes’ crystallographic structures [42]. However, a number of Cc residues located at the back of the molecule also became protected, indicating considerable mobility of the bound Cc. Furthermore, the protection factors for hCc–CcP complex were very small [129], which was taken as an additional evidence for a dynamic interaction. From comparison of the predicted and observed protection factors, Jeng et al. [129] estimated that the crystallographic hCc-CcP binding region must be cleared for 25–40% of the lifetime of the complex. In another NMR study, Moore et al. [131] probed the binding of CcP to Nε,Nε′,13 C-dimethyl lysine derivatives of yCc and hCc by two-dimensional [1H, 13C] COSY spectroscopy. It was found that at least 6 (yCc) or 7 (hCc) lysine residues were involved in the interaction with CcP, which was interpreted as an evidence for the multiple binding geometries in Cc-CcP complexes. Unfortunately, only 3 yCc dimethyl lysine resonances, and none for hCc, could be unambiguously assigned, precluding detailed analysis of the observed binding effects.

3.2.1. PRE NMR spectroscopy

Very recently, the extent of dynamics in yCc–CcP complex was quantified by paramagnetic relaxation enhancement (PRE) NMR spectroscopy [100,101,125,132]. PRE is caused by magnetic dipolar interactions between a nucleus and an unpaired electron of a paramagnetic probe [133,134], which can be introduced into the molecular frame by bioconjugation techniques. Due to the large magnetic moment of the unpaired electron and r−6 distance dependence, protein nuclei located close to the paramagnetic centre experience very large PREs, so that even lowly-populated species can give rise to a measurable effect. For protein complexes in the fast exchange regime, the measured PRE is a population-weighted average of the contributions from all protein–protein orientations and, as such, contains the information on both the specific binding form and the encounter state [133].

In order to map out the conformational space explored by the interacting yCc and CcP molecules, a paramagnetic spin-label (SL) was introduced to 10 different locations, one at a time, on the surface of CcP via an engineered cysteine residue (Fig. 9A), and intermolecular PRE effects on Cc nuclei were measured. Three SLs located close to the crystallographic binding site (N38C, N200C and T288C, shown as red spheres in Fig. 9A) gave rise to PREs, while SLs attached to any of the other seven positions (blue spheres in Fig. 9A) showed no effects [100,125]. Most of the observed PREs (Γ2∗) arise from the dominant form of yCc–CcP complex (Fig. 9B, N200 profile) and can be used to calculate the structure of the complex in solution [100]. However, several Cc regions experience additional paramagnetic effects (highlighted in Fig. 9B). As the experiments were performed in conditions precluding formation of a 2:1 Cc–CcP complex (1=125 mM), the additional PREs (Γ2∗) contain no contribution from Cc bound to the low-affinity site, but rather originate from protein–protein orientations constituting the encounter state [100]. Most of Γ2∗ map out to the N- and C-terminal Cc helices (Fig. 9B). The latter region is similar to that exhibiting large protection factors in the HD exchange study of Yi et al. [130] and experiencing chemical shift perturbations (Δς) upon binding to CcP [59]. However, no Γ2∗ effects or significant Δς were observed for Cc W59 and N60 groups, two residues showing large protection factors in the complex with CcP [130].

Refinement of the observed PREs (Γ2∗) against a simulated ensemble of electrostatically-favourable protein–protein orientations [125] provided an estimate for the population of the yCc–CcP encounter state (p), which was subsequently confirmed by an independent analysis [132]. Thus, it was found that the proteins spend 70% of the lifetime of the complex in the dominant orientation as seen in the crystal structure [42] and 30% sampling multiple binding geometries in the dynamic encounter state [125]. A related study of variant Cc–CcP complexes demonstrated that p can be modulated by interfacial point mutations [101]. In particular, it was shown that R13A Cc mutation leads to a dramatic increase in the population of the encounter state (p = 80%), rendering it the dominant form of the protein complex.

Knowledge of p allowed decomposition of Γ2obs for the wt complex into the contributions from the dominant Cc–CcP form (Γ2p) and the encounter state (Γ2∗), which was used to refine the encounter ensemble [132]. As can be judged from Γ2 plots in Fig. 9B, a combination of PREs from the refined ensemble and the dominant, crystallographic orientation provides a good agreement with the experimental data. In the encounter state, Cc explores an area of CcP surface surrounding the high-affinity, crystallographic binding site (Fig. 9C), corresponding to the most favourable Cc–CcP binding region in the classical BD simulations of Northrup et al. [48]. While sampling multiple binding orientations, Cc undergoes a considerable rotational motion, in agreement with earlier experimental [130,131] and theoretical [48,122] studies. The encounter ensemble is characterised by large separations between redox centres (Fig. 9D), indicating that the overwhelming majority of protein–protein orientations constituting the encounter state are ET-inactive.

When compared to the results of earlier FRET work [126,127], the heme-to-heme distance distribution obtained from PRE analysis appears to be broader and shifted towards higher values (cf. Figs. 8 and 9D). Another, more puzzling, observation is that, contrary to PRE NMR experiments that detect the encounter state at 300 K, FRET...
decay curves at the same temperature exhibit no heterogeneity as would be expected for multiple protein-protein orientations [126,127]. As $\Gamma_2$ PREs are insensitive to fast dynamics (ps-ns timescale) and report mainly on slower motional processes ($\mu$s-range) [134], the fact that encounter PREs could be readily detected suggests that the accompanying dynamics are governed by the latter timescale. Moreover, the lifetime of the encounter state ($\tau^*$) can be calculated from its population ($p = 30\%$) and the lifetime of Cc-CcP complex ($\tau$), either measured directly ($\tau = 1.5-2.5\text{ ms}$) [58] or estimated from the dissociation rate constant ($\tau = 1/k_{\text{off}} = 0.8\text{ ms}$) [59]. The calculated value of $\tau^* = 250-750\mu\text{s}$ indicates that the encounter state dynamics occur on the timescale of a FRET experiment and, given the broad distribution of donor-acceptor distances (heme-to-heme data in Fig. 9D), should give rise to complex FRET decay curves at 300 K. However, no such behaviour was observed experimentally [126,127]. Together with the differences between the distance distributions obtained by the two experimental techniques, this apparent discrepancy can be due to differences in the experimental conditions (in particular ionic strength, $I = 12\text{ mM}$ in FRET work [126,127] and $I = 125\text{ mM}$ in PRE studies [100,125]) or a possible perturbation of the ‘native’ Cc-CcP interaction by Mg-substituted CcP used as a FRET donor.

4. Electron transfer in Cc–CcP complex

4.1. Historical perspective

The discovery of CcP in yeast [1,2] prompted comparisons with plant peroxidases, such as horse radish peroxidase (HRP). Known for a substantial time, HRP is a paradigm example of a prosthetic group-dependent enzyme, specific for its peroxide substrate but much less specific for the co-substrate reductant. In the first published kinetic comparison of CcP and HRP, Chance [135,136] identified the former as always showing first order kinetic behaviour (and hence a comparatively high apparent Michaelis constant, $K_m$, or a weak affinity for Cc), whereas the HRP reaction obeyed zero order kinetics, suggesting an intermediate role for an enzyme co-substrate complex.

Working on a mitochondrial Cc oxidase, Conrad and Smith [137] unexpectedly discovered that the oxidation followed first order kinetics, even at Cc levels sufficient to secure maximal turnover. The explanation was that reduced (Cc(Fe$^{2+}$), substrate) and oxidised (Cc(Fe$^{3+}$), product) Cc had the same binding affinity for the enzyme, indicating product inhibition by ferric Cc. Further, the authors reasoned that if ferric Cc was inhibitory, then so was the ferrous form. Thus, both bound forms of the enzyme were deemed inactive, and the active species was free enzyme, which oxidised ferrous Cc in a bimolecular ‘collision’ process. Beetlestone [3] adapted this model for CcP. Assuming the inhibition by both ferric and ferrous Cc, the behaviour in this kinetic model is still ‘Michaelian’, but the apparent $K_m$ is actually the inhibition constant ($K_i$), while the apparent maximal turnover is the product $k \cdot K_i$, where $k$ is the bimolecular rate constant (in $\text{M}^{-1}\text{s}^{-1}$). Were the inhibition to be eliminated, the turnover would be much larger than the experimentally determined maximal value. The proposed mechanism was hard to refute by any simple kinetic analysis.

Clearly spelled out by Minnaert [138,139], alternative kinetic models for Cc oxidase, especially those involving ‘active’ complex with ferrous Cc and product inhibition by ferric Cc, prompted a re-examination of Cc oxidation by both HRP and CcP. Perhaps in the last such attempt before the discovered complexities of the mitochondrial oxidase made further parallel comparisons unfruitful, Nicholls [4,140] compared both enzymes with Cc oxidase. Contrary to Chance, who had postulated Cc binding to HRP but not CcP (see above), exactly the opposite was found. In particular, it was shown that HRP formed no complex with Cc, and its oxidation involved internal ET from either oxyferryl or porphyrin n-cation radical in CpdI to a more surface-
located site, accessible to a large co-substrate molecule like Cc. In contrast, CcP, like Cc oxidase, seemed to form an ‘active’ complex with ferrous Cc and an inactive one with the ferric protein. However, the identification of the kinetic intermediate with any stable complex formed between the two ferric proteins in the absence of peroxide remained to be demonstrated.

After his fundamental work on Cc oxidase, Yonemitsu [21] developed a new procedure for isolating yeast CcP, which gave a product with much higher activity than that obtained by the original method of Altshul and co-workers [2]. Yonemitsu identified the initial product of the reaction with peroxide as a true Michaelis complex, complex ES [5]. Analogies with both myoglobin and non-specific peroxidases indicated that this intermediate had to be a mixed oxyferryl/radical species, in which the radical was much more stable than in the other heme proteins. Yonemitsu demonstrated the presence of this radical and modified his model [37,141]. In the subsequent studies [6,32,34,49], the apparently functional complex was compared with the inactive, yet stable ferric–ferric and ferrous–ferric Cc–CcP forms, whose affinities were determined by biophysical methods [49].

All the initial kinetic measurements were consistent with a role for a single, ferric or ferrous Cc molecule associated with the enzyme in the catalytic cycle [3,5,34]. In the simplest such model, the binding and dissociation of Cc were fast processes. Therefore, the Cc(Fe²⁺)-CcP complex had the character of a true Michaelis complex, whose binding constant was numerically close to the Michaelis constant for CcP complex had the character of a true Michaelis complex, whose binding constant was numerically close to the Michaelis constant for CcP [6,32,34,49]. The complication, not shared with classical ‘Michaelis-type’ enzymes, was that the full catalytic cycle involved two molecules of Cc and two peroxidase intermediates, CpdI and CpdII (Fig. 10).

The two ‘sides’ of the cycle are essentially symmetrical. If peroxide is present in kinetic excess and the product dissociation is fast, the steady state system at high levels of Cc(Fe²⁺) will contain equal amounts of CpdI-Cc(Fe²⁺) and CpdII-Cc(Fe²⁺) complexes, linked by the limiting ET rates with an ‘average’ Michaelis complex as in Eq. (5):

\[ \text{CpdI} \rightarrow \text{Cc}(\text{Fe}^2\text{+}) \rightarrow \text{CpdII} \rightarrow \text{Cc}(\text{Fe}^2\text{+}) \]  \hspace{1cm} (5)

\[ \text{CcP} \rightarrow \text{Cc}(\text{Fe}^3\text{+}) + \text{H}_2\text{O}_2 \rightarrow \text{CpdI} \rightarrow \text{Cc}(\text{Fe}^2\text{+}) \rightarrow \text{CcP} \rightarrow \text{Cc}(\text{Fe}^3\text{+}) \rightarrow \text{CpdII} \rightarrow \text{Cc}(\text{Fe}^2\text{+}) \]  \hspace{1cm} (6)

If complex formation and dissociation are much faster than the redox reactions, then all the CcP molecules will be associated with Cc even at lower peroxide levels, and the initial peroxide reaction will be with the Cc–CcP complex (Eq. (6)). If the product, Cc(Fe³⁺), also binds and dissociates rapidly, an exponential, Smith-Condor type enzyme kinetics will be observed at all concentrations of the substrate, Cc(Fe²⁺), as is usually the case [3]. For a comprehensive overview of the steady-state kinetics in this system, the reader is referred to the review by Erman and Vitello [7] and the recent book of Dunford [142] on catalases and peroxidases.

4.2. “One site” model

4.2.1. Steady-state kinetics

After early enzymological studies of CcP (Section 4.1.), a number of more recent reports [70,143,146] investigated steady-state kinetics of CcP-catalysed peroxide reduction by both yCc and hCc. It was found that, at Cc saturation and low peroxide concentrations, the reaction between the resting-state CcP and H2O2 is governed by a bimolecular rate constant of 3.5 ± 7 μM⁻¹ s⁻¹, while at higher peroxide concentrations (>200 μM) some enzyme inactivation is observed [143]. At H2O2 saturation and low ionic strength, the initial velocities exhibit biphasic dependence on Cc concentration [70,144,146]. For hCc-CcP, the Michaelis constants for the two phases, K_{M1} and K_{M2}, do not correlate with the dissociation constants for two Cc binding sites, K_{D1} and K_{D2}, and, thus, cannot be interpreted in terms of a simple two-binding site mechanism [143]. For wt yCc-CcP complex, though, there is a good agreement between K_{M1}, K_{D1} and K_{M2}, K_{D2} [70,110]. However, this agreement breaks down for W191F CcP [145], casting doubt on the use of K_{M1} and K_{M2} as a measure of Cc binding affinity at two sites. As the determined rate constants report on a process comprising two one-electron reduction steps each involving Cc binding, intracomplex ET, and dissociation (Fig. 10), the Cc–CcP steady-state kinetics are complicated and, despite promising studies [110,147] attempting to rationalise the K_{D(i)} values in terms of these elementary steps (Section 4.2.5.), remain poorly understood [7].

4.2.2. Stopped-flow kinetics

In order to obtain the individual rate constants for the reduction of CpdI and CpdII (Eqs. (3)–(4), Section 1.3.), transient-state kinetics were studied by stopped-flow techniques [41,107,109,148–153]. Initial accounts of transient CpdI reduction by hCc and yCc at low ionic strength reported biphasic curves, originally interpreted as reduction of CpdI to CpdII, followed by reduction of CpdII to the resting-state enzyme [41,148,149]. However, the second, very slow phase was later shown to be due to small amounts of H2O2 in the working solutions of Cc [109]. The current explanation of these transient kinetics data is that CpdI reduction to CpdII at low ionic strength is too fast to be resolved in the stopped-flow experiment, and the initial phase is due to the reduction of Fe(IV) = O species in CpdII (see below).

To resolve the fast CpdI reduction phase and establish which of the two centres, W191F or Fe(IV) = O, is reduced first, Hahn et al. [150] used two stopped-flow protocols: protocol A, employing an excess of CpdI over Cc and protocol B, utilising excess of Cc. In each case, transients at 416 nm (Cc oxidation) and 434 nm (oxyferryl reduction at Cc isobestic point) were followed. It was shown that at CpdI excess (protocol A), only the former is observed, indicating that Fe(IV) = O is not reduced and Cc oxidation is entirely due to CpdI W191F reduction. At Cc excess (protocol B), both transients are detected, consistent with the subsequent reduction of Fe(IV) = O in CpdI. In protocol A, the decay curve at 416 nm is monophasic in the range of ionic strengths tested; however, only 40% of the expected absorbance change is detected at 1–50 mM, indicating that a large part of the reaction at low ionic strength is too fast to be resolved in this experiment [150]. In protocol B, a biphasic transient at 416 nm, reporting on both CpdI and CpdII reduction steps, and a slow monophasic decay at 434 nm, reporting on Fe(IV) = O reduction in CpdII, are detected. Both CpdI and CpdII reduction steps are described by bimolecular rate constants k_{b1} and k_{b2}, respectively. For hCc-CcP in 2 mM phosphate pH 7.0, k_{b1} = 400–33 μM⁻¹ s⁻¹ and k_{b2} = 30–3.5 μM⁻¹ s⁻¹ were measured at I = 54–154 mM [150]. Based on these results, Millett and co-workers [151] proposed the following kinetic

![Fig. 10. CcP enzymatic cycle.](image-url)
scheme (Fig. 11). In the first step, W191** radical of CpdII (W191**), Fe (IV) = O) is reduced to yield CpdII containing heme oxyferryl Fe(IV) = O group (CpdII). This is followed by a rapid, pH-dependent equilibration between two forms of CpdII and subsequent reduction of W191**, containing CpdII (CpdIIa) to yield the resting-state enzyme. In this scheme, \( k_1 \) and \( k_2 \) are assumed to be equal, and the measured rate constants for the two reduction steps are given by \( k_a = k_1 \) and \( k_b = k_2/(K + 1) \). 

Study of the ionic strength dependence of \( k_a \) and \( k_b \) for yCc and hCc oxidation at pH 7.0 [107] revealed that both rate constants increase with decreasing I and exhibit the same slopes in log (k) = f (1/\( I \)) plots (Fig. 2 in ref. [107]). For both cytochromes, \( k_a \) is an order of magnitude smaller than \( k_b \) at any given I, and both rate constants are consistently larger for yCc than those for hCc. Extrapolating the trends to low I values suggests that CpdII reduction is too fast to be measured at low ionic strength, consistent with earlier findings (see above). A later, more detailed, stopped-flow study by Wang et al. [108] established that for yCc, \( k_a \) varies from 180 \( \mu \)M \(^{-1}\)s \(^{-1}\) at I = 130 mM to 3,000 \( \mu \)M \(^{-1}\)s \(^{-1}\) at I = 100 mM, while \( k_b \) exhibits a more complex behaviour. It initially rises from 27 \( \mu \)M \(^{-1}\)s \(^{-1}\) at I = 310 mM to 250 \( \mu \)M \(^{-1}\)s \(^{-1}\) at 160 mM, then progressively slows down at I = 100 mM (Fig. 8 in ref. [109]). This observation, together with the evidence from photo-induced ET measurements (Section 4.2.3), suggests that at low ionic strength the reaction is limited by Cc(Fe\(^{3+}\)) release from CpdII-Cc(Fe\(^{3+}\)) complex, implying that product dissociation in the first reduction step is the rate-limiting process for the subsequent Fe(IV) = O reduction at low I [109].

4.2.3. Photo-induced electron transfer

To address the mechanistic details of the CcCP catalytic cycle, several groups have studied photo-induced ET in Cc-CCP complex. The idea to use photochemistry to trigger intracomplex ET in Cc-CCP system was initially exploited by Tollin and co-workers [106,154-157], who reported on a series of transient kinetic studies whereby laser flash photolysis is used to reduce flavin to a flavin semiquinone. The latter reduces Cc(Fe\(^{3+}\)) bound to CpdII, and intracomplex ET is monitored. The initial study showed that ET rates to CpdII vary significantly among the different cytochromes used (horse, tuna and yeast iso-2) [154]. The ET kinetics were interpreted using the Poulos and Kraut model [81], and it was concluded that the static model could not account for all aspects of the protein-protein interaction in solution and, thus, the kinetics reflected some average of ET-active species in rapid equilibrium (i.e. a dynamic assembly). Whilst the recognition of dynamics within the complex was with hindsight correct, the ET rates were not in keeping with later studies. The rate constants measured in the flavin system were assumed to be for ET within a 1:1 complex, whereby the Fe(IV) = O site of CpdII was reduced first [154]. This conclusion contrasts sharply with the findings of later stopped-flow and flash photolysis studies (discussed below), demonstrating that the W191** radical is reduced first and at a much faster rate. Given the low reduction potential (E\(^o\)) of flavin/flavin semiquinone system (E\(^o\) ~ -230 mV [154], compared to E\(^o\) = 260 mV for Cc(Fe\(^{3+}\))/Cc(Fe\(^{2+}\)) redox couple [10]), it appears that the reported intramolecular ET rates were limited by the initial reduction of Cc(Fe\(^{3+}\)).

To overcome these limitations, Millett and co-workers developed an alternative photo-induction scheme, harnessing favourable photochemical properties of Ru\(^{3+}\)-bis(bipyridine) derivatives. First, Ru\(^{3+}\) probe is covalently attached to Cc surface via a single lysine or cysteine residue (see ref. [158] for an overview of the photoconjugation techniques used), and the resulting modified protein (Cc-Ru) is purified by standard chromatographic procedures [159]. Photo-excitation of Ru\(^{3+}\) probe generates a strong reducing agent, Ru\(^{3+}\)/Ru\(^{2+}\) (E\(^o\) (Ru\(^{3+}\)/Ru\(^{2+}\)) = -720 mV [159]), which can either decay to the ground state or reduce Cc(Fe\(^{3+}\)), generating a Ru\(^{3+}\)-Cc(Fe\(^{3+}\)) intermediate (Fig. 12). The latter can either return to the ground state by back ET from Cc(Fe\(^{2+}\)) to Ru\(^{3+}\) or transfer an electron to its redox partner such as CpdII. Addition of sacrificial electron donors such as EDTA or aniline prevents the back ET and generates ET-competent Ru\(^{3+}\)-Cc(Fe\(^{3+}\)) species (protocol 1 in Fig. 12) [160,161]. Alternatively, Ru\(^{3+}\)-Cc(Fe\(^{3+}\)) intermediate can oxidise W191 in the resting-state CCP (E\(^o\) (Ru\(^{3+}\)/Ru\(^{2+}\)) = 1.3 V [159]), thereby generating W191** radical (i.e. CpdIIa species) along with the Ru\(^{3+}\)-Cc(Fe\(^{3+}\)) product (protocol 2 in Fig. 12) [108]. The latter strategy allows using W191** reduction in the absence of Fe(IV) = O and H\(_2\)O\(_2\) normally used to generate CpdII [108]. Depending on the attachment site, the ET from Ru\(^{3+}\) to CpdI (Fe\(^{3+}\)) occurs with a rate constant k\(_b\) = 10\(^{-10}\) s\(^{-1}\) [159], enabling measurement of fast ET from Cc(Fe\(^{3+}\)) to CpdII. Among the main criticisms of the Ru-mediated photo-induced ET approach are the observed variability of ET with different Cc-Ru derivatives and a concern that steric hindrance from the introduced bulky probe, as well as surplus positive charge imparted by Ru\(^{3+}\) ion, might perturb the native Cc-CCP interaction. Nevertheless, as shown in what follows, Cc-Ru studies of Millett and co-workers [107-109,147,152,158-163] have provided a wealth of information on the mechanism of the intermolecular ET in Cc-CCP system.

Photo-excitation of Cc-Ru in the presence of CpdII results in intermolecular ET, which is reported by Cc oxidation or Fe(IV) = O reduction and can be monitored spectrophotometrically [160], similarly to what is done in stopped-flow experiments (Section 4.2.2). At all experimental conditions tested, no change in absorbance due to Fe(IV) = O species is detected, yet Cc oxidation is observed [107,109,152,158-160] [159]. This conclusively shows that W191** is the primary reduction site in CpdII, in good agreement with the stopped-flow studies discussed above. Subsequent reduction of Fe(IV) = O can be observed in multiple-flash experiments [160], where re-excitation of Cc-Ru provides additional reducing equivalents for the second ET step. In 2 mM phosphate pH 7.0, depending upon hCc-Ru derivative, the rate constants for the first and second reduction processes are k\(_{etb}\) = 5,200-55,000 s\(^{-1}\) and k\(_{etb}\) = 110-350 s\(^{-1}\), respectively [161], while for yCc modified at position 39 (yCc-Ru39) the values are k\(_{etb}\) = 2 \times 10\(^5\) s\(^{-1}\) and k\(_{etb}\) = 5,000 s\(^{-1}\) [109]. The rate constant k\(_{etb}\) for the reduction of Fe(IV) = O reports on CpdIII - CpdI conversion, followed by the intracomplex ET from Cc(Fe\(^{2+}\)) to W191**. Observation of faster ET in the physiological yCc-CCP complex compared to hCc system parallels the findings of stopped-flow experiments [107]. For all hCc-Ru derivatives, k\(_{etb}\) decreases with increasing ionic strength [161], again in agreement with stopped-flow kinetics [107], while ET rates for yCc-Ru39 are virtually constant in I = 2-100 mM range [109]. At low ionic strength, reduction of W191** with hCc-Ru conjugates exhibits biphasic behaviour [107,152,161], which was taken as evidence for the presence of multiple Cc-CCP binding forms and led the authors to...
suggest that “It is possible that the binding orientation responsible for the slow phase is inactive in electron transfer, and the observed rate constant is governed by the rate of conversion to the fast orientation” [161]. Interestingly, recent PRE NMR studies have shown that most of the Cc-Cp orientations constituting the dynamic encounter state are ET-inactive (Fig. 9D, Section 3.2.1.), lending support to the above hypothesis.

4.2.4. CpdII–CpdId equilibrium

Derived from the stopped-flow and photo-induced ET data, the kinetic model of Millett and co-workers (Fig. 11) postulates that 1) both ET steps take place from Cc(Fe(III)) bound at the high-affinity site to W191 “+” group on CcP and 2) there is a fast equilibrium between two CpdII forms, regenerating W191 “+” radical for the second ET event. Experimental verification of these conjectures has so far provided no unequivocal conclusions. The necessity for the ET to proceed via W191 “+”, as opposed to direct ET to Fe(IV) = O, has primarily been inferred from the results of steady-state and transient kinetics studies [145,164], which reported a 10,000-fold decrease in the rate of Fe(IV) = O reduction for W191F CcP compared to the wt enzyme. Additional evidence for the pivotal role of W191 “+” comes from the recent work of Goodin and co-workers [165–167], who have excised W191-G192-A193 stretch of the proposed ET path in CcP and reconstituted it with a synthetic (N-benzimidazole-propionic acid)-Gly-Ala-Ala peptide (BzGAA). Despite very close structural similarity of the native and BzGAA-reconstituted CcP, the latter is catalytically-inactive (\(k_{\text{cat}} < 0.02\%\) that of the wt enzyme) [167], highlighting the importance of W191 “+” radical for the intermolecular ET. However, the facts that reduction of wt CcP prepared in pure Fe(IV) = O form (i.e. CpdII, species) generates no significant amount of W191 “+” [168], and H175Q/W191F CcP mutant exhibits considerable catalytic activity (ca. 20% that of the wt enzyme) [169] illustrate the possibility of extra-W191 ET.

Early kinetics study of Cpdld reduction by hCc(Fe(III)+) and [Fe(CN)]63− established the presence of two species: one with a “free-radical-like” EPR spectrum (now known to be CpdId, containing W191 “+”) and the other being Fe(IV) compound responsible for the changes in the optical spectra (Cpdld containing heme oxyferryl group, Fe(IV) = O) [40]. Based on the kinetic evidence alone, the authors could not discriminate between mechanisms involving two independent binding sites or two sites in rapid equilibrium; however, for the latter case, the equilibrium constant \(K = [\text{CpdId}] / [\text{CpdId}]_0\) was estimated at different pH values and shown to vary from 1.2 at pH 5 to 0.0 at pH 8.0 [40]. A later stopped-flow study by Millett and co-workers [151] demonstrated that for hCc at 110 mM the rate of W191 “−” reduction, \(k_R\), is virtually independent of pH, while the rate constant for Fe(IV) = O reduction, \(k_P\), steadily decreases from 55 μM−1 s−1 at pH 5.0 to 1.5 μM−1 s−1 at pH 8.0. Similar pH dependence of \(k_R\) and \(k_P\) was observed for yCc [152]. Estimates of the equilibrium constant showed that \(K\) varies from 1.1 ± 0.3 at pH 5.0 to 0.05 at pH 8.0 [151,152], in excellent agreement with the original report [40].

Interestingly, \(K = f(pH)\) dependence fits well to a single ionisation event with \(pK_a = 5.7 \pm 0.1\), suggesting that the equilibrium between Cpdldl and CpdIdl forms can be controlled by a single Cc residue [151]. A similar pH transition was observed by steady-state kinetics and shown to be buffer-dependent, with \(pK_a\) values of 4.0 and 5.5 in phosphate and nitrate, respectively [170–172]. The CcP heme environment contains three titratable groups: D235, located at the proximal side and hydrogen bonded to W191 and heme ligand H175 (Fig. 4), and H52 and R48 residues found in the distal heme pocket. The steady-state kinetics of the wt and D235N CcP exhibit similar pH dependence, which rules out D235 as the source of the observed ionisation event [173]. Study of R48L and R48K Cc mutants suggested that R48 sidechain contributes to destabilisation of the protonated form of H52 and binds nitrate, explaining the observed buffer-dependence [174]. Finally, analysis of H52L CcP identified H52 residue as the group associated with the low pH transition and playing a critical role in Cpdldl stabilisation [172]. Given the similarity of the \(pK_a\) values obtained from the steady-state [170–172] and transient [151] kinetics, it is plausible that the ionisation status of H52 also governs the CpdIIl-CpdIdl equilibrium. Experimental study of the ET properties of Cpdldl in H52-substituted CcP variants could provide a means to verify this hypothesis.

Using stopped-flow kinetics and photo-induced Cc-Ru ET, Liu et al. [152] measured the exchange rate between two CpdIIl forms. To observe intramolecular ET in Cpdll, the authors had to find conditions under which reduction of W191 “+” in Cpdll is much faster than the rate of the subsequent Fe(IV) = O to W191 “+” conversion. This was achieved by lowering the pH, thereby shifting the CpdIIl-CpdIdl equilibrium to the right. Another way to affect this equilibrium was offered by substituting M230 CcP residue thought to stabilise W191 “−” radical. Thus, it was shown that the M230I mutation had little effect on Cpdll W191 “+” reduction, yet impaired intramolecular ET to Fe(IV) = O, significantly decreasing the rate of Cpdll reduction [152]. For the wt CcP, the Cpdll–CpdIdl exchange rate constant, \(k_{\text{ex}}\), was measured at pH 5.0 (\(k_{\text{ex}} = 1.100 \pm 1.000 \) s−1) and pH 6.0 (\(k_{\text{ex}} = 600 \pm 600 \) s−1). Though being higher than earlier estimates obtained at pH 7.0–7.5 [41,168,175], the obtained \(k_{\text{ex}}\) values were argued to be too slow to account for the results of this and earlier works [40,152]. A possible explanation for this discrepancy, yet to be tested experimentally, is that conversion from Cpdll to CpdIdl occurs only in the transition state, with possible stabilisation of W191 “+” radical [168].

If the transition from Cpdll to CpdIdl is the rate-limiting process in Cpdll reduction, the rate constant for the second step, \(k_{\text{etb}}\), should be the same for all Cc-Ru conjugates exhibiting fast reduction of W191 “+” (i.e. large \(k_{R}\)), and remain approximately equal to the Cpdlll–CpdIdl conversion rate, \(k_{\text{etb}}\), which was measured at pH 5.0 (5.7 ± 0.1) at pH 7.0 [109]. However, \(k_{\text{etb}}\) values for hCc-Ru vary from 110 to 350 s−1 [161] and are slightly lower than the \(k_{\text{etb}}\) estimate, suggesting that another process must limit the rate of Fe(IV) = O reduction under these experimental conditions (see Section 4.2.5.). For yCc-Ru39, \(k_{\text{etb}} = 5,000 \pm 1,000 \) s−1 [109] is an order of magnitude higher than \(k_{\text{etb}}\), which is inconsistent with the simple kinetic scheme proposed earlier (Fig. 11). In order to resolve this discrepancy, Millett and co-workers [109] presented an additional mechanism of Cpdll reduction, taking into account Cc binding and dissociation events (Fig. 13).

The reduction of the heme oxyferryl group in CpdIdl proceeds via multiple steps, starting from A and leading to the product H. For simplicity’s sake, the rates of Cc association (\(k_a\)) and dissociation (\(k_d\)) are assumed to be independent of Cc and CcP redox states [109]. Transition from CpdIIl to CpdIdl includes internal ET to W191 group (denoted by \(R\) and formation of Fe(III)−O intermediate \(C\), which is converted to F (CpdIdl) via hydrogen transfer and concomitant water release (C-F step). The same sequence of events (steps B-G) takes place in CpdIdl complex with Cc(Fe(III)+). The exchange constant between A and F (and, by analogy, B and G) is given by \(k_{\text{ex}}\), determined experimentally by Liu et al. [152]. Under the conditions where \(\text{Cc(Fe}^{2+})\) binding is rate-limiting (such as those in the studies of hCc-Ru derivatives [152,161], which used small Cc concentrations and a single equivalent or excess of Cpdll), Fe(IV) = O reduction follows A-C-F-G-H pathway. In this case, the rate constant \(k_{\text{etb}}\) includes the contributions from Cpdlll–CpdIdl conversion steps (A-C-F) and Cc binding (step G-F) steps. Saturating Cc(Fe(III)+) conditions (such as those in the study of Wang et al. [109], employing excess yCc-Ru39 over Cpdll), B-D-G-H or B-D-E-H paths will be followed. According to Wang et al. [109], the latter pathway is strongly favoured as ET from Cc(Fe(II)+) to W191 “−” is faster in D-E than G-H step due to charge neutralisation of W191 “−” radical—cation by Fe(III)−O species [109]. In this case, the rate of the intracomplex ET to Fe(IV) = O is no longer limited by \(k_{\text{ex}}\), but is given instead by \(k_{\text{ex}} = k_{\text{etb}} / (1 + k_{\text{etb}})\), where \(k_{\text{etb}} = [D]/[B]\), which explains the large observed \(k_{\text{etb}} = 5,000 \) s−1 [109].
4.2.5. Role of the low-affinity binding site

The final aspect of the “one-site” ET model concerns the role of the low-affinity Cc binding site, known to contribute to Cc-CcP interaction at low ionic strength (Section 2.2.). To address this issue, Millett and co-workers studied the interaction of Cc(Fe$^{3+}$) with the pre-formed CpdI-Cc(Fe$^{3+}$) complex at low ionic strength by photo-induced ET kinetics [162] and stopped-flow spectroscopy [153]. The authors found no evidence for ET from Cc bound at the low-affinity site, yet observed the dissociation rate constant of Cc bound to the high-affinity site, $k_{d}$, increases from $k_{d}=5$ s$^{-1}$ in the 1:1 complex to $k_{d}=600$ s$^{-1}$ ($I=4$ mM [162]) or $k_{d}=2,000$ s$^{-1}$ ($I=10$ mM [153]) upon binding of the second Cc molecule. The value of $k_{d}=5$ s$^{-1}$ is smaller than $k_{d}=180$ s$^{-1}$ obtained at $I=10$ mM in the NMR study of Yi et al. [58], possibly due to the contribution of 2:1 complex, unaccounted for in the NMR work [162]. At $I>74$ mM the effect of the second Cc binding event is no longer measurable [162], consistent with observed destabilisation of the 2:1 complex at high ionic strength (Section 2.2.). These findings confirmed the “substrate-assisted product dissociation” model proposed before [58,106,127] and suggested that “... the rate-limiting step in enzyme turnover is product dissociation below 150 mM ionic strength, and intracomplex electron transfer to the oxyferryl heme above 150 mM ionic strength.” [162].

The latter conclusion was corroborated by the steady-state kinetics work of Miller et al. [110,147], who showed that the change in the ionic strength dependence of $k_{cat}$ and the deviation from hyperbolic kinetics at $I \leq 110$ mM are caused by a change in the rate-limiting step and established that at $I \leq 110$ mM the enzyme turnover is limited by Cc-CcP dissociation. At low ionic strength, a simple scheme assuming only 1:1 complex formation cannot account for the observed kinetics with $k_{cat} \gg k_{d}$. Thus, to take into account the contribution of Cc molecule bound at the low-affinity site, Miller [110] performed numerical simulations of the kinetic model containing 1:1 and 2:1 Cc-CcP complexes using the published values for the elementary reaction steps (Fig. 13). The developed model predicts enzyme turnover rates at the high-affinity binding site and reproduces the experimentally observed ionic strength dependence of $k_{cat}$. In agreement with the results of Millett et al. [153,162], Cc bound at the low-affinity site is shown to be ET-inactive [110], suggesting that “The existence of this unreactive binding site is probably serendipitous, since it does not contribute to catalysis at physiological ionic strength.” [147].

From the above kinetic evidence, the “one-site” ET model can be summarised as follows. Both reduction steps involve ET from Cc bound at the high-affinity, crystallographic site to W191$^{+}$ of Ccp. There is no direct ET to Fe(IV) = O, but rather fast equilibration between CpdII and CpdIII, regenerating W191$^{+}$ for the second reduction step. Cc bound at the low-affinity site exhibits no ET activity, but enhances dissociation of Cc bound at the high-affinity site. Cc dissociation after the first ET event or the subsequent CpdIII$^{-}$ conversion is the rate-limiting step for CpdI reduction at the low and high ionic strength, respectively.

4.3. “Two-sites” model

It was demonstrated that under some experimental conditions, especially low ionic strength, CcP catalysis exhibits biphasic behaviour with respect to Cc concentration [33,70], which presented one of the earliest complications in the analysis of the kinetic curves. Kang et al. [33] analysed the data as simply involving two independent, equally accessible sites, perhaps associated with the heme oxyferryl and radical species, leading to the first proposal of a 2:1 Cc-CcP stoichiometry. Soon after that, Erman and co-workers [70] interpreted similar data in terms of a single binding site, but with two distinct Cc reactions and, hence, purely kinetic biphasicity; yet later they found that either explanation could account for the results [143].

Hoffman and co-workers have presented the most compelling evidence for an ET-active 2:1 complex in a series of studies exploiting the photochemistry of Zn-porphyrins [43–45,176]. The authors showed that intermolecular ET in Cc-ZnCcP complex can be studied by substituting the heme in either CcP or Cc with a Zn-porphyrin (ZnP) and phototriggering its redox chemistry [9]. Fig. 14 illustrates an ET cycle for ZnCcP bound in a 1:1 complex with Cc. Photo-excitation of the ground-state ZnCcP leads to the formation of a triplet state, $^{3}$ZnCcP, which in the absence of Cc(Fe$^{3+}$) returns to the ground state with a decay rate ($k_{D}$) of $\sim 120$ s$^{-1}$ [43]. When bound to Cc(Fe$^{3+}$), $^{3}$ZnCcP can either decay directly to the ground state with a rate constant ($k_{0}+k_{b}$), where $k_{b}$ is the rate of non-redox quenching, or undergo an intermolecular, forward ET with a rate constant $k_{f}$ to yield a charge-separated intermediate, I ($D^{-}\cdot A^{+}$). The latter returns to the ground state via a back ET from the bound Cc(Fe$^{3+}$) to the ZnP-based π-cation radical (ZnP$^{+}$) with a rate constant $k_{0}$ (Fig. 14). The ZnP system differs from the Cc-Ru ET setup (Section 4.2.3) in that the photo-initiated ET event proceeds in a non-physiological direction, i.e., from CcP to Cc. However, the back ET reaction mirrors the physiological flow of electrons from Cc(Fe$^{3+}$) to CpdIII. Early criticisms of this approach raised concerns that back ET occurs directly between two heme groups, bypassing formation of W191$^{+}$ species and, thus, is non-physiological [7,147,153,162]. Moreover, despite the formal equivalence of the two processes, the kinetics of Cc(Fe$^{3+}$) reduction by $^{3}$ZnCcP and CcP(Fe$^{3+}$) are different, suggesting that ZnP substitution perturbs the native Cc-CcP interaction [7].

Titrating ZnCcP with Cc from several species – hCc and fungal Cc (fCc) from Candida krusei, Pichia membranifaciens, or iso-1 form from S. cerevisiae – Stemp and Hoffman [43] observed that the quenching
constant, $k_q = k_0 + k_{so}$, continued to increase past the point where CCP should be saturated with Cc if the binding was 1:1. In the case of FCC, it was found that the intermediate species I did not accumulate until the second Cc molecule was bound. This was taken as an evidence for the ET-inactive 1:1 complex, with the observed quenching entirely due to a non-redox process (i.e. $k_q = k_0$), and suggested that the ET takes place only at the second binding site on CCP [43]. The latter conclusion was reinforced by the observation that increasing the ionic strength eliminates the quenching attributed to ET, consistent with disruption of a reactive, electrostatic 2:1 Cc-CCP complex. A slightly different scenario was observed for hCc. A build up of I occurred at the 1:1 stoichiometry and rose synchronously with $k_q$ upon binding of the second Cc molecule. The 2:1 stoichiometry was thus inferred but, in contrast to FCC complexes, both binding sites on CCP were ET-active, with the second site being more ET efficient [43]. The quenching titrations were also used to obtain binding constants for Cc(Fe
sup+3-)–ZnCCP complexes. At low ionic strength, the first Cc binds tightly ($K_d<1 \mu M$), followed by a weaker binding of the second molecule ($K_d>100 \mu M$). Moreover, it was shown [43] that dissociation rate constants, $k_{off}$, of both ferrous and ferric Cc from the second binding site are highly sensitive to the ionic strength and $k_{off}$ (Cc(Fe
sup+3-))$< k_{off}$ (Cc(Fe
sup+2-)), consistent with release of the product, Cc(Cc
sup+3+), during the catalytic cycle and a higher affinity for the substrate, Cc(Fe
sup+3+).

An inverse titration was also reported, whereby quenching of the photo-initiated triplet state of ZnP-substituted hCc (ZnPCCP) with resting-state Cc(Fe
sup+3-) was monitored [44]. The $k_q$ was found to increase up to [CCP]/[ZnPCCP] = 1:2 and then decrease with further addition of CcP(Fe
sup+3-) quencher [44]. These results were consistent with two Cc molecules binding simultaneously, and the ET quenching being much more efficient in a 2:1 complex. By way of a further variation to this system, Hoffman and co-workers introduced a reverse protocol, whereby the quencher was held at a fixed concentration and the photo-excitic ZnP probe titrated [45]. The results from this study again compounded the 2:1 stoichiometry of the complex at low ionic strength. The findings of detailed photochemical studies of Hoffman and co-workers were summarised in a following model [9]. At Cc : CCP ratios of > 1, a second distinct binding site is required to explain the quenching data, with binding affinity to the Site 2 binding lower than that to Site 1 by a factor of 1,000. For the CcP complexes with FCC, the quenching data are consistent with ET activity occurring only at Site 2, whereas hCc–CCP complex exhibits ET at both sites, but ca. 1,000 times faster at Site 2 under low ionic strength conditions.

The binding of two Cc molecules to CCP, and the notion that ET took place predominately at Site 2 in the physiological complex, raised further questions about the interplay between bound Cc molecules and ET in the complex. These were addressed by the introduction of a copper–substituted Cc, Cc(Cu
sup+2+), into the ZnP ET photocycle [176]. This redox-inert inhibitor was designed to examine the ‘back’ ET step ($k_{off}$) and probe cooperativity between Cc molecules, i.e. whether Cc bound at the high-affinity site enhances ET activity at Site 2 [176]. Back ET displayed multiphasic kinetics, which was suggested to be due to gating by conformational interconversion [43,177,178]. As ET occurs predominately in the 2:1 complex, this means that the charge-separated intermediate I has the 2:1 stoichiometry [ZnP
sup+1+CCP: Cc(Fe
sup+3-): Cc(Fe
sup+2-)], which was potential able to oxidise W191, which opens the possibility of a two-step, “hopping” return to the ground state as indicated in Fig. 14 ($k_{off}$ path). The W191F CcP mutant [178] showed that $k_q$ in W191F CcP-yCc is much slower compared to that in the wt 1:1 complex. In the mutant complex, the population of I steadily increases up to a 1:1 Cc–CCP ratio, contrasting sharply with the findings for the wt complex, where I is not observed at equimolar protein ratio either in solution [43] or the crystalline state [96]. The W191F CcP mutant cannot be oxidised by ZnP
sup+3+, hence the return to the ground state is slow, allowing I to accumulate [179]. From this study it is clear that W191 does have a role in the ZnPCCP system: it acts as an ET mediator “short circuiting” the direct heme-heme ET through a two step “hopping” process (Fig. 14). In the case of hCc, the observation of I in both solution and the crystal at 1:1 stoichiometry suggests that $k_q$ is decreased with respect to the optimally coupled yCc–CCP complex.

4.4. Electron transfer in covalently cross-linked complexes

Study of the ET in covalent Cc–CCP complexes offers a way to address the functional properties of low- and high-affinity Cc binding sites one at a time, without complications arising from Cc interaction at the other site. In particular, cross-linking Cc with CCP allows blocking one Cc binding site, while leaving the other one free to interact with an exogenously added Cc. Early cross-linking efforts (discussed in detail in Section 2.3.1.) were complicated by heterogeneity of the products, ambiguity of the cross-linking sites, and possible inactivation of the resulting covalent complexes by an excess of a...
cross-linking reagent. Even in the case of Cc derivatives modified with a cross-linker at specific residues, uncertainty of the corresponding attachment site on CcP often precluded the unequivocal interpretation of the kinetics data [146]. Perhaps the most conclusive study of this kind was performed by Bisson and Capaldì [83], who showed that K13 Cc–CcP cross-link was inactive towards exogenously added Cc, suggesting that functionally-active Cc binding site is blocked in the covalent complex.

In a conceptual breakthrough obviating most of the earlier problems, Poulos and co-workers [180,181] prepared several homogenous cross-links, covalently coupled via an intermolecular disulfide bond between single-cysteine Cc and CcP variants. Three covalent complexes were prepared: Complex I (C102 Cc–E290C CcP), with ‘back-to-front’ Cc–CcP coupling; Complex II (K73C Cc–E290C CcP), mimicking the crystallographic binding geometry; and Complex III (K79C Cc–K149C CcP), targeting the putative low-affinity Cc binding site. Complex I showed no ET from the covalently linked Cc, most likely due to a large separation between the redox centres, while Complex II exhibited fast intramolecular ET with a lower limit of 800 s⁻¹ [180]. Moreover, it was demonstrated that W191F CcP mutation leads to a 1000-fold decrease in the intramolecular ET rate of the Complex II, suggesting that the ET proceeds via reduction of W191[F] in CpdI [181]. In addition, Complex II was inactive towards exogenously added yCc and hCc at high ionic strength and protein concentrations, but restored the wt catalytic activity at a decreased yCc concentration and low ionic strength [181]. At the same time, Complex III showed virtually no ET between the cross-linked Cc and CcP, but remained catalytically active towards the exogenously added cytochromes, indicating that the blocked Cc binding site is not functionally important [181].

In a follow-up work, Guo et al. [97] studied an alternative Complex II (A81C Cc–V197C CcP) by X-ray crystallography, stopped-flow spectroscopy, and steady-state kinetics. With an rmsd of 2.82 Å in the crystal structure. Furthermore, detailed analysis of the ionic-strength dependence of the steady-state kinetic parameters for Cc K79C–V5C CcP cross-link revealed strong electrostatic repulsion between the covalently-attached Cc and the Cc molecule non-covalently bound to the high-affinity site at low ionic strength [184].

5. Species-specific differences in Cc–CcP interaction

As mentioned above (Section 2.1.), yCc binding to CcP is stronger than that of hCc in a broad range of experimental conditions. Numerous ITC studies [60–66] have shown that hCc–CcP and yCc–CcP complexes exhibit markedly different binding thermodynamics (Table 3 and Fig. 15). The former is characterised by an unfavourable enthalpy (ΔH) and large, favourable entropy term (ΔS –TΔS), while the latter is driven by favourable enthalpic and entropic contributions. Binding enthalpy of hCc–CcP complex changes little in the range of temperatures studied [60], while ΔH of yCc–CcP shows a clear temperature dependence [62]. Given that binding constants of both complexes are virtually temperature-independent [46,60,62], this reveals a strong enthalpy-entropy compensation in yCc–CcP, but not hCc–CcP, interaction. Furthermore, comparison of the heat capacity changes (ΔCp = dΔH/dT) in hCc-CcP (ΔCp ≈ 0 [60]) and yCc–CcP (ΔCp = −216 ± 62 cal·mol⁻¹·K⁻¹ [62]) complexes – and the fact that yCc binding to CcP shows ΔCp > 0 and ΔS > 0, a signature of the hydrophobic effect [185] – indicates that the hydration properties of the two interfaces are different, with likely larger desolvation in yCc–CcP complex.

The ionic strength dependence of the thermodynamic parameters is also different for the two complexes (Fig. 15). For hCc–CcP, ΔH is virtually independent of I, while -TΔS steadily decreases with increasing salt concentration. In contrast, both thermodynamic parameters of yCc–CcP are highly sensitive to I, exhibiting opposing trends indicative of enthalpy-entropy compensation. As a consequence, the hCc–CcP binding constant shows stronger ionic strength dependence than that of yCc–CcP (Fig. 15, top panel), in agreement with an early steady-state kinetics work [33]. Another remarkable difference in salt sensitivity of the two complexes is illustrated by steady-state and stopped-flow kinetics studies [110,143,149], showing that hCc–CcP is more active at low ionic strength, while the opposite is true for yCc–CcP (see Tables 6 and 7 in ref. [7]).

Another set of species-specific differences in Cc–CcP interaction has been revealed by NMR spectroscopy. It was shown that Cc binding to CcP can be conveniently monitored by spectral changes of hyperfine-shifted heme resonances of both proteins [55–58,69,93] and, with the advent of isotope labelling techniques, chemical shift perturbations of the backbone amide nuclei of Cc [59]. With characteristic, strong resonances located outside of the diamagnetic envelope, Cc heme methyls 3 and 8 have proven to be particularly useful probes. In the entire range of solution conditions and protein concentrations tested, heme methyls of hCc exhibit a single set of resonances that change their position upon binding to CcP, indicating that free and bound proteins are in fast exchange on

### Table 3

Thermodynamic parameters for Cc binding to the high-affinity site on CcP. ΔH and -TΔS (in kcal/mol) were obtained by ITC. Note a good agreement among the values obtained from different studies.

<table>
<thead>
<tr>
<th>Cc</th>
<th>ΔH</th>
<th>-TΔS</th>
<th>Conditions and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCc</td>
<td>2.8 ± 0.8</td>
<td>-10.9 ± 0.3</td>
<td>pH 6.0 = 20 mM 26 °C [60]</td>
</tr>
<tr>
<td>bCc</td>
<td>2.7 ± 0.3</td>
<td>-10.9 ± 0.6</td>
<td>pH 7.0 = 18 mM 25 °C [63]</td>
</tr>
<tr>
<td>yCc</td>
<td>6.2 ± 0.3</td>
<td>-1.9 ± 0.4</td>
<td>pH 6.0 = 20 mM 25 °C [62]</td>
</tr>
<tr>
<td>yCc</td>
<td>-2.6 ± 0.1</td>
<td>-5.5 ± 0.1</td>
<td>pH 6.0 = 20 mM 25 °C [64]</td>
</tr>
<tr>
<td>yCc</td>
<td>-2.3 ± 0.4</td>
<td>-5.3 ± 0.5</td>
<td>pH 6.0 = 20 mM 25 °C [65]</td>
</tr>
<tr>
<td>yCc</td>
<td>-1.6 ± 0.1</td>
<td>-5.7 ± 0.2</td>
<td>pH 6.01 = 115 mM 30 °C [66]</td>
</tr>
</tbody>
</table>

* K72 is trimethylated. Removing the methyl groups increases ΔH by 3.6 ± 0.1 kcal/mol, but, due to enthalpy-entropy compensation, has no effect on ΔS [64].
the chemical shift time scale [55–57,93]. A lower-limit value for the dissociation rate constant of hCc–CcP complex was estimated from binding-induced chemical shift perturbations5 at \( k_{\text{off}} \approx 200 \text{ s}^{-1} \) [55] and later corrected to \( k_{\text{off}} = 1133 \pm 120 \text{ s}^{-1} \) [56]. In contrast to hCc–CcP, the exchange regime of yCc–CcP binding depends on experimental conditions [186], with high temperature, ionic strength, and protein concentration promoting fast exchange and \( k_{\text{off}} \) values varying from 180 s\(^{-1}\) at low ionic strength [58] to >1200 s\(^{-1}\) at higher salt concentrations [59]. The slow-to-intermediate exchange observed in diluted protein solutions at low 1 is manifested by two sets of heme methyl resonances—their of free and CcP-bound yCc [57,58,93,186]. However, as explained by Moench et al. [93], the difference in exchange behaviour of hCc and yCc does not necessarily reflect the difference in the corresponding dissociation constants, but rather stems from larger binding shifts of yCc.6

NMR chemical shift perturbations (\( \Delta \delta \)) of yCc resonances by binding to CcP are larger than those of hCc [57,93]. In fact, the \( \Delta \delta \) values for hCc in complex with CcP are similar to those induced by salt [93,187] or binding of polyglutamate [188], suggesting low specificity of hCc–CcP interaction. Given a good correlation between the \( \Delta \delta \) size and the population of the dominant protein–protein orientation in CcP complexes [101], smaller \( \Delta \delta \) values suggest that hCc–CcP is more dynamic than yCc–CcP. This finding agrees well with the results of hydrogen-deuterium exchange experiments [129,130] (see Section 3.2.), which showed smaller protection factors for hCc–CcP than yCc–CcP complex, and FRET work of McLendon et al. [127], who observed a broader distribution of heme-to-heme distances in hCc–CcP system, implying its higher mobility.

However, in their studies of covalently cross-linked Cc–CcP complexes, Erman and co-workers [91,93] found that cross-linking yCc and CcP produces a heterogeneous mixture of 1:1 adducts, while reaction between hCc and CcP yields a single covalent product, with an NMR spectrum highly similar to that of the native, non-covalent hCc–CcP complex. This seems to suggest that yCc interaction with CcP is more dynamic than that of hCc—a finding which is difficult to rationalise given the above evidence to the contrary.

X-ray crystallographic analyses [42,96] have established that yCc–CcP and hCc–CcP adopt different binding geometries in the crystal (Fig. 6). In order to verify whether these structural differences are preserved in solution, Miller et al. [147] prepared A193C/C128A CcP variant modified with a bulky thiol reagent, which would block crystallographic binding site of yCc, but not perturb that of hCc. The authors observed a large, 20 to 100-fold decrease in transient and steady-state ET rates for yCc reaction with the modified CcP, but only minor effects for hCc, whereby confirming the similarity of the crystallographic and solution Cc–CcP structures. Moreover, the rate constant for the first ET step in modified yCc–CcP system is similar to that in the native, unmodified hCc–CcP complex [147], suggesting that the introduced steric substituent steers yCc away from its crystallographic binding site and into a binding orientation similar to that observed in hCc–CcP X-ray structure.

Using Ru-labelled Cc derivatives to study photo-induced ET to CcP, Millett and co-workers [163] demonstrated that, at low ionic strength, structural differences between hCc–CcP and yCc–CcP complexes have clear functional implications: the ET in hCc–CcP, but not in yCc–CcP system, is “gated” by conformational dynamics of protein molecules. Based on their experimental evidence, the authors proposed that hCc–CcP complex consists of a major, ET-inactive form (ca. 90%) and a minor form (ca. 10%) favouring fast ET, and that the conformational change between the two forms is the rate-limiting step in ET process. Furthermore, the authors suggested that the major and minor forms correspond, respectively, to crystallographic hCc–CcP and yCc–CcP binding geometries [163], implying that hCc must undergo rotational and translational motion relative to CcP in order to adopt an ET-active conformation.

Another study from Millett’s group [107] showed that, at high ionic strength, CcP mutations designed to probe the crystallographic yCc–CcP binding site have the same effect on ET kinetics of both yCc and hCc complexes, suggesting that the ET-active yCc–CcP and hCc–CcP forms are the same. Given that both hCc–CcP structures reported so far [42,96] have been solved at low ionic strength, independent verification of this hypothesis must await hCc–CcP structure determination at a high salt concentration.

6. Physiological role of Cc–CcP complex

Based on the catalytic activity of Cc–CcP complex in vitro, it was proposed that it acts as an antioxidant, peroxide-scavenging system in vivo [189], and a later study confirmed the role of CcP as a general catalyst for H\(_2\)O\(_2\) reduction in aerobic yeast mitochondria [190]. Recently it was shown that CcP is not essential for viability and respiration in S. cerevisiae [191,192], yet it contributes to the resistance against exogenous oxidative stress in vivo [193–195], and its expression level is increased in aerobic conditions [191]. The fact that haploid yeast cells deficient in CcP (cpp1\(^{-}\)) are able to sustain aerobic metabolism [191,192] suggests a compensatory role of other antioxidant enzymes such as superoxide dismutase, catalase, and glutathione reductase in mitigating the oxidative stress associated with aerobic respiration [192]. In addition to, and independent of, its antioxidant activity, CcP participates in oxidative stress signalling [192,193,196] and appears to be part of a complex cellular defence network against the reactive oxygen species (ROS) [192,196]. An in vivo study showed that, just like in S. cerevisiae, CcP contributes to the resistance against ROS in a fungus Cryptococcus neoformans [197]. This finding suggests that the physiological function of Cc–CcP complex is not unique to yeast and, considering the high conservation of
proteins’ primary sequences among lower and higher eukaryotes [10,197], might be a general feature of eukaryotic response to ROS. Interestingly, recent clinical studies have shown that CpP-containing eye drops significantly accelerate corneal epithelial healing after phototherapeutic keratectomy in mice [198,199]. Presumably, CpP mitigates wound healing by neutralising elevated levels of ROS associated with local inflammatory responses [199]. Finally, another recent work has demonstrated that, in addition to its antioxidant function, Cc–CcP couple is involved in electron shuttling in the mitochondrial intermembrane space [200]. It was shown that a flavin-dependent sulphhydryl oxidase, Erv1, reduces Cc and utilises molecular oxygen as an electron acceptor to generate hydrogen peroxide, which is subsequently reduced to water by CpP using reducing equivalents provided by Erv1-reduced Cc. Thus, Cc acts as an electron shuttle between Erv1 and CpP, effectively coupling the two redox cycles [200].

7. The end of the road?

During the past seventy years (Fig. 1), the Cc–CcP complex has been extensively characterised by a range of biochemical and biophysical techniques and become a paradigm for the study of transient protein interactions and long-range, biological ET. The trophy cabinet of this complex features X-ray and solution Cc–CcP structures, experimental map of the conformational space explored by the interacting proteins, biophysical characterization of Cc binding to the low-affinity site, and a working kinetic model, explaining most of the catalytic properties. After seven decades of research and hundreds of papers written on the subject, what is left to discover in this – ad nauseam studied for some, fascinating for others – system? Below we outline several unknowns that are likely to shape Cc–CcP research in the coming years.

Most of the structural and biophysical studies have been performed on Cc(FeS²⁺)-CcP(Fe³⁺) and Cc(FeS⁺³)-CcP(Fe³⁺) systems, corresponding to the initial association and final dissociation complexes, respectively (Fig. 10). In addition, CpP-CN was used as CpdP surrogate, mimicking its spin and coordination states. However, the question remains whether these structural mimics are representative of the transient Cc(FeS⁺³)-CcP and Cc(FeS²⁺)-CcP species active during the enzyme turnover. As X-ray structures of CpdP [201–203] and CpdDl [203] are virtually identical to that of the resting-state enzyme, and considering that CpP heme group is buried, minimising the electrostatic effects of Fe(IV) = O and W191²⁺ centres on binding, such an assumption appears sound, albeit experimentally unproven.

Despite the progress made in the study of the low-affinity binding site (Sections 2.2. and 2.3.4.), the weak, salt-sensitive 2:1 Cc–CcP complex has so far resisted unequivocal structural characterization. Although this is not integral to understanding the Cc–CcP catalytic and binding properties under the physiologically-relevant (high ionic strength) solution conditions, the Cc–CcP story is by no means complete without the structure of the 2:1 complex.

The best working model, dubbed here “one-site ET” (Section 4.2.), accounts for most of the findings obtained by steady-state and transient kinetics and is consistent with numerous structural and biophysical studies. However, the obligatory role of W191²⁺ in the reduction of Fe(IV) = O in the CpdP and the underlying rapid CpdDl–CpdDl equilibrium are still controversial issues (Section 2.4.2.). Thus, the possibility of alternative ET pathways, not involving W191²⁺ centre, remains open. Using a combination of site-directed mutagenesis and kinetics techniques [23,25,151], it was shown that CpP residues Y39, Y42, W51, M172, H181, W223, and Y229 are not involved in intermolecular ET from Cc, which leaves a number of other methionine and tyrosines residues as suitable candidates for a transient radical location.


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