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Review

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

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ABSTRACT

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.

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 H_2O_2 to water requires two reducing equivalents from ferrous Cc (Eq. (1)) and involves formation of Cc–CcP protein complex [3–6].

$$H_2O_2 + 2Cc^{2+} + 2H^+ = 2Cc^{3+} + 2H_2O \quad (1)$$

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 b_2 (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[1] 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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[1] The amino-acid numbering used in this work is based on the sequence alignment with horse heart Cc that generates a negative numbering for the first five residues (Fig. 3).

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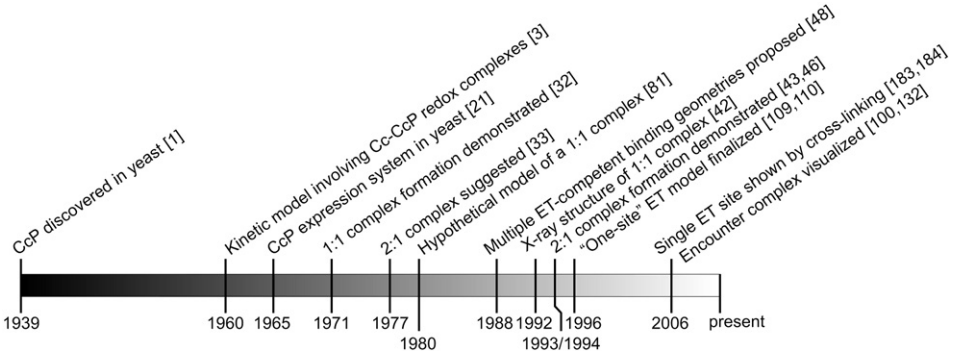

Fig. 1. Cc–CcP research timeline. Selected milestones indicated.

by covalent thioether bonds with two cysteine residues from the canonical CXXCH sequence (Fig. 2B). The heme contains a low-spin (S = ½), 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 $N_{\varepsilon 2}$ atom of H18, and the S_{δ} 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 (yCc) and non-physiological binders (cytochromes from organisms other than yeast) exhibit distinct differences in their interaction with CcP (see Section 5). In this respect, the variation in charge distribution (Fig. 3), resulting in a 2.4-fold difference in dipole moments of yeast and horse Cc,[2] is particularly noteworthy, given the importance of electrostatics in Cc–CcP interaction.

1.2. 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 $N_{\varepsilon 2}$ 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 ($S = {}^5/_2$), 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 = ½) form of CcP 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^{2+}) 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].

$$\mathrm{CcP(Fe^{3+}) + H_2O_2 = CpdI + H_2O} \tag{2}$$

$$\mathrm{CpdI + Cc(Fe^{2+}) = CpdII + Cc(Fe^{3+})} \tag{3}$$

$$\mathrm{CpdII + Cc(Fe^{2+}) = CcP(Fe^{3+}) + Cc(Fe^{3+})} \tag{4}$$

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 ($W191^{+\bullet}$) [37–39]. CpdII, a one-electron reduction product of CpdI, contains either an Fe(IV) = O ($CpdII_F$) or $W191^{+\bullet}$ ($CpdII_R$) species, depending on experimental conditions [40,41]. The last two

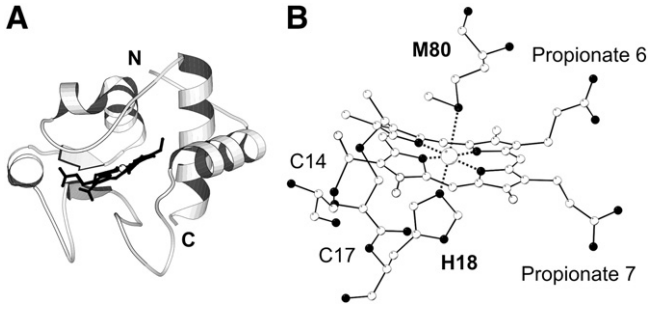

Fig. 2. Three-dimensional structure of yCc. (A) Overall fold of the protein; the heme is shown in sticks, and the protein termini are indicated by the labels. (B) The view of the heme binding site showing the axial ligands, H18 and M80 (bold labels), and two cysteine residues, C14 and C17, covalently linked to the heme group; heme propionates are indicated. The molecular coordinates of ferric yCc were taken from the PDB entry 2YCC [209]. Figs. 1 and 3 were generated with MOLSCRIPT [210].

[2] The dipole moments of ferric yCc (μ = 522.9 D) and hCc (μ = 215.7 D) were calculated with MacroDox 4.5.3 [123,204] at pH 7.0 I = 0.1 M t = 25 °C, using Tanford–Kirkwood partial charge assignments for titratable groups [205]. The angle between the two dipole vectors is 10°.