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Reduction Potential of Yeast Cytochrome *c* Peroxidase and Three Distal Histidine Mutants: Dependence on pH

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Abstract

The pH dependence of the Fe(III) reduction potential, E^0 , for yeast cytochrome *c* peroxidase (yCcP) and three distal pocket mutants, CcP(H52L), CcP(H52Q), and CcP(R48L/W51L/H52L), has been determined between pH 4 and 8. E^0 values at pH 7.0 for the yCcP, CcP(H52L), CcP(H52Q), and CcP(R48L/W51L/H52L) are -189, -170, -224, and -146 mV, respectively. A heme-linked ionization in the reduced enzyme affects the reduction potential for yCcP and all three mutants. Apparent pK_A values for the heme-linked ionization are 7.5 ± 0.2 , 6.5 ± 0.3 , 6.4 ± 0.2 , and 7.0 ± 0.3 for yCcP and the H52L, H52Q, and R48L/W51L/H52L mutants, respectively. A cooperative, two-proton ionization causing a spectroscopically-detectable transition was observed in the ferrous states of yCcP, CcP(H52L) and CcP(H52Q), with apparent pK_A values of 7.7 ± 0.2 , 7.4 ± 0.1 and 7.8 ± 0.1 , respectively. These data indicate that: (1) the distal histidine in CcP is not the site of proton binding upon reduction of the ferric CcP, (2) the distal histidine is not one of the two groups involved in the cooperative, two-proton ionization observed in ferrous CcP, and (3) the proton-binding site is not involved in the cooperative, two-proton ionization observed in the reduced enzyme.

Keywords

Cytochrome *c* Peroxidase; Distal Histidine mutant; Spectra; Reduction Potential

1. Introduction

The Fe(III) reduction potentials of heme proteins can vary by over a volt at neutral pH[1-3]. The potentials are influenced by axial heme ligation, the local heme environment, solvent exposure of the heme, and chemical modification of the porphyrin ring [4-10]. Electrostatic effects can play a major role in modulating the reduction potential by stabilizing or destabilizing the Fe(III) heme relative to the Fe(II) heme since these two redox states differ in the formal net charge at the heme core. Early measurements of the reduction potentials of hemoglobin [11], myoglobin [12], cytochrome *c* [13], and horseradish peroxidase (HRP) [14] showed that all were dependent upon pH indicating that the oxidation-reduction reactions were linked to proton equilibria. Yamada and Yamazaki [15] investigated the proton balance between five redox states of HRP and found that electron transfer was

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accompanied by proton binding in four of the five reactions investigated. Proton binding accompanied the reduction of ferric HRP, HRP Compound I, HRP Compound II, and HRP Compound III. Proton binding was also observed during reduction of cytochrome *c* peroxidase (CcP) Compound I and CcP Compound II [16]. Proton coupling to electron transfer has been noted in other redox systems and has led to the concept of charge compensation at the active sites in redox proteins [17,18].

Since the original study on HRP, the pH dependence of the Fe(III) reduction potential for several other peroxidases have been determined including two turnip peroxidase (TPO) isozymes [19], yeast CcP [20], lignin peroxidase (LPO) isozyme H1 and the Mn-dependent peroxidase (MPO) isozyme H4 from *Phanerochaete chrysosporium* [21]. All show heme-linked ionizations in the reduced form of the enzyme with the apparent pK_A values ranging between 5.0 and 7.6. Heme-linked ionizations with similar pK_A values also affect the absorption spectra of the ferrous peroxidases. The spectral perturbations in the ferrous state are small for HRP [15], the two TPO isozymes [19], LPO and MPO [21], but quite large for CcP [20]. The large spectral transition in ferrous CcP is associated with the conversion of the five-coordinate heme found at acidic pH to a six-coordinate heme at alkaline pH and involves a cooperative, two-proton ionization that allows coordination of the distal histidine, His-52, to the heme [22-24].

The identity of the group that binds the proton upon reduction of ferric peroxidase has been the subject of debate since the original studies on HRP with initial suggestions involving one of the axial heme ligands or the side chain of a nearby amino-acid residue, generally ascribed to either an imidazole or carboxylate group [15,19-21]. With determination of the crystal structure of CcP [25] and the homology of the heme binding sites in the peroxidases [26], speculation has centered on the distal histidine as the most likely site for redox-linked proton binding [17,21,22], although this is not universally accepted [24,27].

Meunier *et al.* [18] have tested the hypothesis that the distal histidine (His-42) in HRP is the site of the redox-linked proton binding by investigating the redox, spectroscopic, and ligand binding properties of three HRP mutants, R38L, F41A, and H42L. Meunier and coworkers found that the F41A mutant had little effect while the absorption spectrum and Fe(III) reduction potential of the H42L and R38L mutants had a weaker pH dependence than wild-type HRP. These data support the view that His-42 is the primary protonation site upon reduction and that protonation of His-42 affects the reduction potential and absorption properties of the enzyme. Meunier and colleagues also suggest that Arg-38 is involved through modulation of the pK_A of His-42 and that, in the absence of His-42, charge compensation upon reduction of the heme iron can occur by binding protons to sites further removed from the heme, leading to the weak pH dependence of the reduction potential observed for the H42L and R38L mutants.

In light of the weak pH dependence of the Fe(III) reduction potential in HRP(H42L) [18], the differences in acid-alkaline transition in ferrous CcP compared to ferrous HRP [15,20], and the disagreement on the identity of the proton binding site that affects the Fe(III) reduction potential in CcP [22,24], we have investigated the pH dependence of the Fe(III) reduction potential and spectroscopic properties of the ferrous enzyme for three distal histidine mutants of CcP, H52L, H52Q, and a triple mutant, R48L/W51L/H52L, which we name CcP(TriLeu). The pH dependence of the reduction potential for the three mutants is similar to that of wild-type CcP, except for a small shift in the pK_A for the redox-linked site of protonation. The simplest interpretation of the data is that His-52 is not the site of protonation upon reduction of ferric CcP.

2. Materials and Methods

2.1. Materials

Yeast CcP (yCcP) was isolated from Red Star baker's yeast using the methods previously described [28]. The recombinant CcP (rCcP) system used in this study has been described previously [29]. CcP(H52L), CcP(H52Q), and CcP(TriLeu) were constructed using the Stratagene QuikChange mutagenesis kit and sequenced in both directions to assure that only the intended mutations were made. Expression and purification of rCcP and the CcP mutants were performed as previously described [28,29]. The expression system removes the N-terminal methionine and the isolated rCcP has the same amino acid sequence as baker's yeast CcP, yCcP.

Between pH 4.0 and 5.5, buffers were 0.010 M acetate with sufficient KH_2PO_4 to adjust the ionic strength to 0.100 M. Between pH 5.5 and 8.0, the buffers were mixtures of KH_2PO_4 and K_2HPO_4 with a total ionic strength of 0.100 M.

Stock solutions of enzyme ($\sim 500 \mu\text{M}$) in buffer were centrifuged at $13,700 \times g$, 5°C for 15 minutes to remove any insoluble material. CcP and CcP mutant concentrations were determined spectrophotometrically using previously determined extinction coefficients [30].

2.2. Spectra of ferrous enzymes

Spectral properties of the ferrous proteins were determined by first measuring the absorption spectrum of the ferric enzyme, then reducing the ferric enzyme with solid sodium hydrosulfite and measuring the spectrum of the reduced enzyme. A Hewlett Packard model 8452A diode array spectrometer (2 nm resolution) was used to determine all spectra.

2.3. Reduction potential determination

The Fe(III) reduction potentials, E^0 , were determined for yCcP and the three CcP mutants using a photochemical reduction method adapted from Mauro *et al.* [31] and Choudhury *et al.* [32] as described previously [30]. The heme Fe(III) reduction potentials are based upon the reduction potential of either riboflavin [33], safranin O [34], or indigo carmine [34]. The reduction potentials of all three dyes are dependent upon pH and the appropriate value was used to calculate E^0 for the Fe(III) heme at each pH.

3. Results

3.1. Heme binding site and location of mutations

Figure 1 shows the heme environment in CcP [25]. Arg-48, Trp-51, and His-52 define the distal heme pocket. His-52 is critical for rapid oxidation of CcP by hydrogen peroxide and the enzyme is essentially inactivated when His-52 is replaced by a non-polar amino acid residue [35]. Hydrogen bonding through His-181 and heme propionate-7 allows for communication between the distal and proximal heme pockets. His-181 is also hydrogen bonded to Asp-37. His-175 is coordinated to the heme iron, defining the proximal heme pocket. Asp-235 hydrogen bonds to both His-175 and Trp-191. All three CcP mutants used in this study replace His-52 with a residue incapable of binding protons, although the H52Q mutant can participate in hydrogen-bonding interactions.

3.2. Spectroscopic properties of the Fe(III) state of CcP and the CcP mutants

The spectroscopic properties of both the ferric and ferrous forms of the enzyme must be known in order to use the photochemical reduction technique to determine the redox potential of CcP and its mutants. CcP and its mutants are generally stable between pH 4 and 8. The spectrum of the Fe(III) form of CcP(H52L) near pH 4, 6, and 8 is shown in Figure 2.

An on-line Supplementary Data file accompanying this article shows similar spectra for rCcP, CcP(H52Q), and CcP(TriLeu) in Figures S1, S2, and S3, respectively. The spectra of the ferric enzyme and the three mutants are similar, characteristic of a predominantly five-coordinate, high-spin heme (5c-hs) between pH 4 and about 7 and showing the beginning of an alkaline transition above pH 7. The ratio of the absorbances at the Soret maximum and in the δ band near 380 nm is used to monitor the 5c-hs to 6c-ls transition in CcP and its mutants. The absorbance ratio, A_{406}/A_{384} , for CcP(H52L) is shown in the lower panel of Figure 2.

Extensive studies of the alkaline transition in yCcP [36] and in CcP(H52L) [37] have shown that the transition is a complex, multi-step process, the slowest of which occurs over many hours. The pH dependence of the initial spectral changes can be characterized by an apparent pK_A value, which is 8.6 ± 0.3 for yCcP [36] and 8.3 ± 0.2 for CcP(H52L) [37]. Apparent pK_A values for the acid-alkaline transition for the CcP variants used in this study are designated pK_{A2} and listed in Table 1. The spectrum of CcP(TriLeu) is more dependent upon pH than the H52L and H52Q mutants and three transitions are apparent between pH 4 and 8. The three pK_A values are listed in Table 1 and representative spectra are shown in the Supplementary Data file, Figure S3.

3.3. Spectroscopic properties of the Fe(II) state of CcP and the CcP mutants

The electronic absorption spectra of ferrous heme proteins are of two basic types, that of a 5c-hs heme and that of a 6c-ls heme. The spectrum of wild-type ferrous yCcP varies with pH, going from a 5c-hs form at low pH to a 6c-ls form at high pH [20]. The high-spin/low-spin transition is associated with a cooperative, two-proton ionization that has an apparent pK_A of 7.7 ± 0.2 [20]. There is good evidence that His-52 coordinates to the heme iron in the 6c-ls species and deprotonation of His-181 is involved in the cooperative ionization [22,24].

The spectrum of ferrous rCcP near pH 4, 6, 8, and 10 is shown in Figure 3 and it is similar to that previously reported for yCcP [20]. Between pH 4 and ~ 7.5 , rCcP has a spectrum typical of a 5c-hs ferrous heme protein with α , β , and Soret bands occurring at 588, 558, and 440 nm, respectively. As the pH increases, the Soret band shows a 12 nm blue-shift to 428 nm and the α and β bands shift to 560 and 538 nm, respectively, band positions characteristic of a 6c-ls ferrous heme group. The lower panel in Figure 3 shows the ratio of absorbances at the Soret maxima for the 6c-ls and 5c-hs forms as a function of pH. This ratio is essentially constant between pH 4 and 7.5, increasing steeply above pH 7.5. The data between pH 5 and 10 was fit to Eq. 1. A_1

$$A = (A_1 + A_2([H^+]/K_A)^n) / (1 + ([H^+]/K_A)^n) \quad (1)$$

and A_2 are the low and high pH limits for the absorbance ratio, K_A is the acid dissociation constant, and n is the minimum number of protons participating in the cooperative transition. The best-fit values for the apparent pK_A and n are 8.1 ± 0.2 and 1.6 ± 0.2 , respectively. In this study, the absorbance spectrum was not extensively studied above pH 8.5 and we attribute the low value of n to insufficient data at the higher pH values. The value of n gives the minimum number of protons involved in a cooperative transition so at least two proton-binding sites are involved in the alkaline transition in rCcP. The more extensive study of the alkaline transition in yCcP [20] found an n value of 2.0.

The spectrum of ferrous CcP(H52L) at pH values near 4, 6, and 8 are shown in Figure 4. The spectral changes in ferrous CcP(H52L) as a function of pH are much less dramatic than

those of rCcP. The visible bands show only modest variations in intensity between pH 4 and 8 while the Soret maximum shifts, going from 440 nm at low pH to 434 nm at pH 8. The spectra indicate that H52L remains primarily 5c-hs between pH 4 and 8.3. Although the alkaline transition does not appear to involve a significant change in heme ligation, the perturbation of the Soret band is influenced by a cooperative, ionization. Fitting the alkaline transition to Eq. 1 gives best-fit values of 7.4 ± 0.1 and 2.1 ± 0.2 for the apparent pK_A and n , respectively, Table 1.

The pH dependence of the spectrum of ferrous CcP(H52Q) is similar to that of CcP(H52L) (Figure S4, Supplementary Data), with a cooperative, two-proton ionization influencing the spectrum at alkaline pH. Best-fit values for pK_{A2} and n are 7.8 ± 0.1 and 2.0 ± 0.3 , respectively, Table 1.

The spectrum of ferrous CcP(TriLeu) is constant between pH 5 and 8 (Figure S5, Supplementary Data). The Soret maximum occurs at 428 nm, suggesting that the TriLeu mutant is 6c-ls in this pH region. The TriLeu mutant is susceptible to acid denaturation below pH 5 and only the beginning of an alkaline transition is observed as the pH is increased above 8. The apparent pK_A for the alkaline transition must be >8.4 , too high to be relevant to the reduction potential studies described below.

3.4. pH dependence of the reduction potential for CcP and the CcP mutants

In order to validate the photoreduction methodology, the reduction potentials for an authentic yCcP sample were determined between pH 4 and 8 using photoreduction and compared with the 1978 results [20], which used potentiometric measurements. An example of the raw data and analysis for the photoreduction method is described in the Supplementary Data file. The experimentally determined values for $E^{0'}$ at pH 7 are -189 ± 2 mV using the photoreduction method compared to -194 ± 2 mV determined by potentiometry [20].

The pH dependence of the reduction potential for yCcP determined by the two methods is shown in Figure S6 in the Supplementary Data file. The pH dependence of $E^{0'}$ was fit to Eq. 2

$$E^0 = E^{0'} + 59.2 \log([H^+] + K_R) \quad (2)$$

with E^0 and K_R adjustable parameters. The potentials are given in mV at 25°. E^0 is the potential extrapolated to 1 M $[H^+]$ assuming no other protonation steps are involved in the reaction and K_R is the acid dissociation constant for the group that binds the proton in the reduced form of the enzyme. Best-fit values pK_R are 7.3 ± 0.1 for the potentiometric technique and 7.5 ± 0.1 for the photoreduction method. The $E^{0'}$ values at pH 7 and the pK_R values are listed in Table 1.

The pH dependence of $E^{0'}$ for the three mutants is shown in Figure 5. CcP(TriLeu) is less stable at acidic pH than the other mutants and $E^{0'}$ could only be measured between 5 and 8. The shape of the potential curves for the mutants is similar to that of wild-type CcP, decreasing with a slope of about -59 mV per pH unit below pH 6, with a positive inflection in the curve near pH 7, and reaching a plateau value above pH 7. Experimentally determined $E^{0'}$ values at pH 7.0 are -170 ± 2 mV, -224 ± 2 mV, and -146 ± 2 mV for the H52L, H52Q, and TriLeu mutants, respectively. Fitting the pH dependence of the reduction potential for the three mutants to Eq. 2 gives best-fit values for pK_R of 6.5 ± 0.3 , 6.4 ± 0.2 , and 7.0 ± 0.2 for the H52L, H52Q, and TriLeu variants, respectively.

Since the pH dependence of E^0 for all three mutants lacking His-52 have a similar pH dependence as wild-type enzyme, these data provide strong evidence that His-52 is not the site of protonation upon reduction of the ferric enzyme to the ferrous state.

4. Discussion

The original observation that reduction of the Fe(III) heme in HRP is accompanied by binding of a proton to the reduced enzyme between pH 4 and 7 [14,15] indicates that the proton affinity of a basic group near the heme is significantly perturbed upon reduction of the enzyme. The proton-binding site must be unprotonated in the ferric enzyme but protonated in the ferrous enzyme between pH 4 and 7. Reduction potential measurements indicate that the apparent pK_A of the proton-binding site is 7.38 in the reduced HRP [14] and 7.5 in ferrous CcP, Table 1. The pK_A values of the heme-linked ionization in reduced HRP and CcP suggest a nearby histidine residue.

Determination of the crystallographic structure of CcP [25], which showed both histidine and arginine residues in the distal heme pocket, reinforced the idea that the proton-binding site is the distal histidine. The positive charges on the ferric heme and the nearby arginine residue can significantly lower the apparent pK_A of the distal histidine. There is indirect, but compelling evidence that the pK_A s of His-52 in CcP and His-42 in HRP are both below 4 in non-binding buffer systems [28,39-42].

Just as with the ferric state, there are no direct determinations of the pK_A for the distal histidine in ferrous peroxidases. Indirect determinations have been made based on pH-dependent properties of the ferrous enzyme including changes in the UV-visible absorption spectrum [15,20,22], Resonance Raman spectrum [24,43,44], and CO binding rate [45,46]. The UV-visible spectrum of ferrous HRP isozyme C has a small perturbation in the alkaline region with an apparent pK_A of 7.07 [15]. The spectroscopic changes in ferrous CcP are much more dramatic, Figure 3. Ferrous CcP undergoes a conversion between a five-coordinate, high-spin (5c-hs) form at low pH to a six-coordinate, low-spin (6c-ls) form of the heme at high pH with apparent pK_A of 7.7 ± 0.2 for yCcP [20] and 8.1 for rCcP, Table 1. Moreover, the 5c-hs to 6c-ls transition is associated with a cooperative, two-proton ionization. Studies on several CcP mutants, including this work, provide strong evidence that the distal histidine is coordinated to the heme iron in the 6c-ls form of ferrous CcP. All CcP mutants studied to date are converted to the 6c-ls form of the ferrous enzyme at alkaline pH except those that lack a distal histidine [22].

It has generally been assumed that the acid-alkaline transition in ferrous peroxidase detected by the reduction potential measurements and by spectroscopic techniques is the same transition and most investigators have suggested that the transition involves deprotonation of the distal histidine, both in HRP and CcP. However, there is growing evidence that neither of these assumptions may be correct [24]. This study provides strong evidence that the proton-binding site detected in the reduction potential measurements is not the distal histidine since all three mutants lacking His-52 show a transition in the ferrous state with an apparent pK_A ranging between 6.4 and 7.0. We also find that the acid-alkaline transition observed in the UV-visible spectrum of the ferrous state is not coincident with the proton-binding transition detected in the reduction potential measurements for the three mutants with apparent pK_A values differing by 0.9, 1.4, and <1.4, for the H52L, H52Q, and TriLeu mutants, respectively, Table 1.

This study clearly shows that His-52 is not the site of proton binding upon reduction of ferric CcP and is not involved in the cooperative two-proton ionization in ferrous CcP. If His-52 is not the proton-binding site, there are only a limited number of other sites that can

fulfill the requirements for the critical proton-binding site. The binding site must be near the heme since the state of protonation affects the reduction potential of the heme and the redox state of the heme affects the binding affinity of the site. Potential proton-binding sites within ~ 10 Å of the heme iron, other than His-52, are Arg-48, His-175, His-181, Asp-235, and the two heme propionate groups, Figure 1 [25]. There are also five water molecules that interact with these groups and which may affect the apparent heme-linked ionizations. These waters are numbered 348, 535, 595, 596, and 648 in the refined structure of yCcP [25]. Four of the five water molecules are shown in Figure 1. CcP mutants have been constructed for all of the listed amino acid residues and data are available providing initial characterization of the ferric and ferrous forms of the mutants including their ferrous CO complexes. Below, we consider the evidence for and against each of the six sites (four amino acid residues and the two heme propionates) listed above as the binding site responsible for proton uptake upon reduction of the ferric enzyme.

Arg-48 is not a good candidate for the proton-binding site. Arg-48 interacts with multiple waters, is located near the bottom of the solvent-access channel in CcP [25], and its pK_A is thought to be relatively unperturbed in both redox states of the enzyme. It is most likely protonated over the entire pH-stability region of CcP in both redox states of the enzyme. It is certainly difficult to imagine that the pK_A of the guanidinium group could be less than 4 in the ferric enzyme, a requirement for the proton-binding site.

The carboxylate group of heme propionate-7, His-181, and Asp-37 form a hydrogen-bonded (H-bonded) cluster near the heme iron, Figure 1. Heme propionate-7 is H-bonded to His-181 and to water-348. His-181 is also H-bonded to Asp-37. The protonation state of the heme propionates can affect the reduction potential in heme proteins [8] and the cluster of acidic and basic groups in CcP near heme propionate-7 could be a site for differential proton binding in the reduced and oxidized states. Unfortunately there is little information concerning the acid-base equilibria in this cluster. The conformation within the crystal structure of ferric CcP near neutral pH suggests that His-181 is protonated and positively charged with the carboxylates of both propionate-7 and Asp-37 ionized and negatively charged. The three residues together give a net negative charge, which could balance the net positive charge on the Fe(III) heme core. Upon reduction of Fe(III) to Fe(II), adding a proton to the cluster would minimize electrostatic interactions in this region of the protein.

Heme propionate-6 does not appear to be a good candidate for the proton-binding site in ferrous CcP. The carboxylate group of propionate-6 is near the bottom of a small, water-filled crevice on the surface of CcP [25]. Propionate-6 H-bonds to the backbone NH group of Lys-37 and to three water molecules. The pK_A of propionate-6 in both ferric and ferrous CcP should be similar to its pK_A in water due to its interaction with the water at the surface of CcP.

His-175 and Asp-235 in the proximal heme pocket form another H-bonding cluster that could provide a proton-binding site that is responsive to changes in the redox state of the heme iron. Since the imidazole side-chain of His-175 is directly bonded to the heme iron its proton affinity will be greatly altered. In addition, the proximal heme pocket is well insulated from the solvent and electrostatic interactions with the ferric heme can further perturb the pK_A values of both His-175 and Asp-235. The available structural and spectroscopic data strongly support H-bonding between the carboxylate of Asp-235 and the ND1 hydrogen of the neutral imidazole side-chain of His-175. The net negative charge on the Asp-235/His-175 grouping would neutralize the net positive charge on the Fe(III) heme, making this region of the protein net neutral. Upon reduction of the ferric iron, proton binding to the Asp-235/His-175 cluster would maintain electrical neutrality in this region of the protein. The proton would bind to the carboxylate group of Asp-235 and to be consistent

with the reduction potential data, the pK_A of Asp-235 would have to be less than 4 in the ferric enzyme and ~ 7.5 in the ferrous enzyme. This seems reasonable in light of the strong driving force to maintain electric neutrality in a hydrophobic region of the protein interior.

The above considerations suggest that Asp-235 in the Asp-235/His-175 cluster is the most likely candidate for differential proton binding between the ferric and ferrous states of CcP. In the ferric enzyme, the carboxylate group of Asp-235 is ionized and the negatively-charged state is stabilized by H-bonding through the hydrogen atom bound to ND1 of His-175 and by electrostatic interaction with the positively charged ferric heme iron. The electrostatic stabilization would be lost upon reduction of the heme iron and the apparent pK_A of Asp-235 would increase. Proton binding at Asp-235 upon reduction of the ferric heme would provide an explanation for the difference in pK_A values for the proton-binding site and for the cooperative, two-proton ionization in the ferrous enzyme, which we suggest is due to the cooperative interaction of heme propionate-7 and His-181. The cooperative ionization is accompanied with His-52 coordination to the ferrous iron in CcP, Figure 3, but not in the mutants lacking His-52, Figures 4, S4, and S5. His-52 has to be unprotonated when it coordinates to the heme iron giving an upper limit of 7.7 for the pK_A of His-52 in ferrous CcP. In the Supplementary Data, we provide additional support for the hypothesis that Asp-235 is the site of proton-binding upon reduction of ferric CcP by a reconsideration of the published literature.

5. Conclusions

We have shown that: (1) the distal histidine in CcP is not the site of proton binding upon reduction of the ferric enzyme, (2) the distal histidine is not involved in the cooperative, two-proton ionization observed in ferrous CcP, and (3) the proton-binding site is distinct from the groups involved in the cooperative, two-proton ionization in the reduced enzyme. With elimination of His-52 as the redox-dependent proton-binding site, Asp-235 appears to be the next best candidate based upon a consideration of all likely proton-binding sites within 10 Å of the heme iron.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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6. Abbreviations

Mutations are indicated by the one-letter code for the amino acid residue in the wild-type protein, followed by the residue number and the one-letter code for the amino acid residue in the mutant protein.

CcP	generic abbreviation for cytochrome <i>c</i> peroxidase.
yCcP	wild-type CcP isolated from baker's yeast
rCcP	cloned recombinant CcP expressed in <i>E. coli</i> with the exact amino acid sequence as yCcP
CcP(MI)	cloned recombinant CcP expressed in <i>E. coli</i> that differs from yCcP by having a Met-Leu dipeptide fused to the N-terminus and two point mutations, T53I and D152G
HRP	horseradish peroxidase, generally referring to the most abundant isozyme, isozyme C
E^{0'}	standard reduction potential at any defined pH
NHE	normal hydrogen electrode

Figure 1.

Heme environment of yCcP [25]. Arg-48, Trp-51, and His-52 define the distal heme pocket. His-175 is coordinated to the heme iron and defines the proximal heme pocket. Asp-235 is hydrogen bonded to both His-175 and Trp-191 in the proximal heme pocket. His-181 H-bonds to heme propionate-7. Four of the five invariant water molecules in the heme pocket are shown, waters 348, 535, 648 and 595. Water-596 is not shown but H-bonds to water-595 and is 2.9 Å away from NE1 of His-52.

(color for on-line version) DiCarlo, Vitello, Erman "Reduction Potential of Yeast Cytochrome *c* Peroxidase and Three Distal Histidine Mutants: Dependence on pH.

(black and white for print version) DiCarlo, Vitello, Erman "Reduction Potential of Yeast Cytochrome *c* Peroxidase and Three Distal Histidine Mutants: Dependence on pH.

Figure 2.

pH dependence of the ferric CcP(H52L) spectrum. Top Panel: Thin line – pH 4.12, Thick line – pH 5.98, Dashed line – pH 8.14. Bottom Panel: Solid points - The absorbance ratio A_{408}/A_{384} . Solid line – Non-linear least squares regression - best-fit line.

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Figure 3.

pH dependence of the ferrous CcP spectrum. Top Panel: Thin solid line – pH 4.17, Thick Line – pH 6.06, Dashed line – pH 8.10, Dashed-dotted line, pH 9.87. Bottom Panel: Solid Points - The absorbance ratio A_{428}/A_{440} . Solid line – Best-fit line.

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Figure 4. pH dependence of the ferrous CcP(H52L) spectrum. Top Panel: Thin solid line – pH 4.14, Thick Line – pH 5.89, Dashed line – pH 8.23. Bottom Panel: Solid Points - The absorbance ratio A_{434}/A_{440} . Solid line – Best-fit line.
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Figure 5.

pH dependence of the reduction potential (vs NHE) for three CcP mutants. Solid upright triangles – CcP(H52Q). Open Circles – CcP(H52L). Solid inverted triangles – CcP(TriLeu). The solid lines are the best-fit lines to Eq. 2 of the text. The dashed line is the best-fit line for the pH dependence of E'^0 for yCcP. Experimental condition: 0.100 M ionic strength, 25°C.

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Table 1

pH Dependent Transitions in Ferric and Ferrous CcP and Three CcP Mutants.

Protein	Redox State	Spectroscopic Transitions			Redox Transitions	E^0 , at pH 7 ^a (mV)
		pK _{A1}	pK _{A2}	pK _{A3}		
yCcP	Fe(III)		8.6 ± 0.3 ^b			-194 ^c , -189
rCcP	Fe(III)		8.6 ± 0.1			
H52L	Fe(III)		8.3 ± 0.2 ^d			-170
H52Q	Fe(III)		7.7 ± 0.2			-224
TriLeu	Fe(III)	4.3 ± 1.3	7.0 ± 0.3	8.3 ± 0.8		-146
yCcP	Fe(II)	5.7 ± 0.2 ^c	7.7 ± 0.2 ^{c,e}	9.4 ± 0.2 ^c	7.5 ± 0.1	
rCcP	Fe(II)	<4	8.1 ± 0.2 ^e			
H52L	Fe(II)	<4	7.4 ± 0.1 ^e		6.5 ± 0.3	
H52Q	Fe(II)	5.6 ± 0.1	7.8 ± 0.1 ^e		6.4 ± 0.2	
TriLeu	Fe(II)	4.4 ± 0.2	>8.4		7.0 ± 0.2	

^aPotentials reported vs NHE.

^breference [36].

^creference [20].

^dreference [37].

^ecooperative, two-proton ionization.