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Effect of Active Site and Surface Mutations on the Reduction Potential of Yeast Cytochrome *c* Peroxidase and Spectroscopic Properties of the Oxidized and Reduced Enzyme

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Abstract

The reduction potentials of 22 yeast cytochrome *c* peroxidase (CcP) mutants were determined at pH 7.0 in order to determine the effect of both heme pocket and surface mutations on the Fe(III)/Fe(II) redox couple of CcP, as well as to determine the range in redox potentials that could be obtained through point mutations in the enzyme. Spectroscopic properties of the Fe(III) and Fe(II) forms of the mutant enzymes are also reported. The mutations include variants in the distal and proximal heme pockets as well as on the enzyme surface and involve single, double, and triple point mutations. A spectrochemical redox titration technique used in this study gave an $E^{\circ'}$ value of -189 mV for yeast CcP compared to a previously reported value of -194 mV determined by potentiometry [C.W. Conroy, P. Tyma, P.H. Daum, J.E. Erman, *Biochim. Biophys. Acta* 537 (1978) 62–69.] Both positive and negative shifts in the reduction potential from that of the wild-type enzyme were observed, spanning a range of 113 mV. The His-52 $\rightarrow$ Asn mutation gave the most negative potential, -259 mV, while a triple mutant in which the three distal pocket residues, Arg-48, Trp-51, and His-52, were all converted to leucine residues gave the most positive potential, -146 mV.

Keywords

Cytochrome *c* Peroxidase; CcP Mutations; Spectra; Reduction Potentials

Introduction

Proteins with heme groups have a wide variety of functions including electron transfer, oxygen transport and storage, catalysis utilizing either hydrogen peroxide or molecular oxygen as oxidants, ligand binding, signal transduction, and control of gene expression. These various functionalities are a manifestation of the capacity of the heme group itself to exhibit a wide range of chemical reactivity, the ability of the iron to change oxidation state, and the ability of the protein matrix surrounding the heme to modulate the electrochemical characteristics of the heme iron. The functional properties of a heme protein are sensitive to the redox characteristics of the heme iron with the cytochromes cycling between the Fe(II) and Fe(III) states and the heme enzymes, normally beginning their catalytic cycles in the Fe(III) state, capable of cycling between both higher and lower oxidation state intermediates.

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The redox potentials of heme proteins can vary over a very wide range of values. For example, the Fe(III) reduction potentials (E° values at pH 7, 1 Atm, 25 °C) of the known c-type cytochromes span a range of more than 1 V. The positive end of this range is +640 mV for cytochrome c552 from *Thiobacillus ferrooxidans* while the negative end is at -400 mV for cytochrome c3 from *Desulfovibrio vulgaris* [1–3]. Factors that have been shown to affect heme reduction potentials include substituents on the heme ring, axial heme ligation, the local heme environment, and solvent exposure of the heme group [4–9]. Environmental effects can either stabilize or destabilize the Fe(III) state relative to the Fe(II) state due to the differences in net charge of the heme core. The Fe(II) heme (exclusive of porphyrin substituents) is neutral while the Fe(III) heme has a net charge of +1.

All heme peroxidases are similar in that they all have the ability to react with H_2O_2 to form an oxidized enzyme intermediate and it is this heme intermediate that subsequently oxidizes a substrate [10–11]. The main difference among the peroxidases is the enzyme specificity for the oxidizable substrate. Oxidizable substrates include indoles, phenols, aromatic amines, lignin, manganese, halide ions, and other proteins such as cytochrome *c* [10–11]. It is likely that individual heme peroxidases have reduction potentials that are specific for their catalytic activity. Among the peroxidases, horseradish peroxidase has one of the more negative Fe(III) reduction potentials, -270 mV at pH 7, 30°C [12] while myeloperoxidase has one of the more positive potentials, +29 mV [13]. Modulation of the redox potentials in the heme peroxidase could allow for greater specificity in response as well as increased electron transfer efficiency.

Cytochrome *c* peroxidase (CcP) is a detoxification enzyme, catalyzing the reduction of hydrogen peroxide by cytochrome *c* [14,15], eq 1. CcP was the first heme enzyme to have its

three-dimensional structure determined by x-ray crystallography [16,17] and hence has served as a model heme enzyme to investigate structure/function relationships in this class of heme protein. A long-term goal in the field of heme protein chemistry is to elucidate the structural determinants of reactivity. If this goal is realized, then heme proteins can be designed to carry out specific functions, even functions not found in nature. An example of such a function would be to utilize hydrogen peroxide for specific hydroxylation and/or epoxidation reactions.

Hydroxylations and epoxidations are normally carried out by monooxygenases, which utilize molecular oxygen as the oxidant incorporating one oxygen atom into the substrate and reducing the other to water [18]. This four-electron redox reaction requires the use of cofactors such as NADH to provide two of the electrons in the overall catalytic cycle. Design of enzymes capable of carrying out hydroxylation and epoxidation reactions utilizing hydrogen peroxide, a so-called ‘peroxygenase activity’, would remove the need for the cofactors. However, hydrogen peroxide is a strong oxidant and tends to oxidize both the heme and amino acid residues such as tryptophan, tyrosine, and methionine unless there is a rapid turnover of the higher oxidation-state iron species. One consideration in designing specific peroxygenases would be to tune the redox potential of the enzyme to carry out ferryl oxygen transfer with minimal oxidative damage to the enzyme. Another example would be to design heme proteins to participate in nano-scale electronic devices where both self-assembly and redox tuning may be important. These are both long-range goals, not yet within reach. The immediate goal of this study is to investigate the effects of active site and surface mutations on the Fe(II)/Fe(III) redox couple in CcP to provide a more complete view of how structural alteration can be used to tune the redox potential of this model heme enzyme.

Materials and Methods

Materials

CcP(yst) was isolated from Red Star baker's yeast using the methods previously described [19]. Mutant CcP was obtained from two different recombinant protein systems: CcP(MI) [20] and CcP(eco) [21,22]. CcP(MI) includes two additional amino acids at the N-terminus of the mature protein. These amino acids are methionine at the -2 position, which allows for the expression of the protein in the bacterium, and isoleucine at the -1 position. In addition, amino acids 53 and 152 are isoleucine and glycine respectively in the CcP(MI) mutant, whereas in the native baker's yeast CcP these positions are occupied by threonine and aspartate respectively. For CcP(eco) the end product of bacterial expression results in no additional N-terminus amino acids and the sequence of the resulting enzyme is identical to that of CcP(yst).

A potassium phosphate buffer, pH 7.0, 0.100 M ionic strength was used for all experiments. The concentration of oxidized riboflavin stock solutions were determined spectrophotometrically using an extinction coefficient of $9.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 466 nm [23]. Solutions containing riboflavin were immediately shielded from light using medium-gauge aluminum foil. Stock solutions of enzyme (~500 M) were made by dissolving small amounts of crystalline or lyophilized sample in potassium phosphate buffer, pH 7.0. Stock solutions were centrifuged at 14,000 rpm, 5 °C for 15 min to remove any insoluble material. CcP and CcP mutant concentrations were determined spectrophotometrically using appropriate extinction coefficients, Table 1.

Spectra of Ferrous Enzymes

The extinction coefficients of the ferric state of CcP and all mutants used in this study have been determined using the pyridine hemochromogen method [24]. Spectral properties of the ferrous state of all proteins was determined between 400 and 700 nm by first measuring the absorption spectrum of the ferric enzyme, then reducing the ferric enzyme with solid sodium hydrosulfite and determining the absorption spectrum of the reduced enzyme. Spectra were measured with a Hewlett Packard model 8452A diode array spectrometer with a resolution of $\pm 2 \text{ nm}$.

Reduction Potential Determination

The Fe(III) reduction potentials, E^0 , were determined at pH 7.0 for CcP and all CcP mutants using a photochemical reduction method adapted from Choudhury *et al.* [21] and Mauro *et al.* [25]. In this method, oxidized riboflavin is photoreduced, followed by redox equilibration between the riboflavin and CcP (or CcP mutant). EDTA serves as a sacrificial electron donor and is the ultimate source of the reducing equivalents. Standard 1 cm quartz cells with septum-sealed tops were used for the photochemical titrations. Stock solutions of EDTA, riboflavin, and buffer were deoxygenated by vigorously bubbling with water-saturated argon gas (99.998% pure) for a minimum of 90 min. A small aliquot of CcP was deoxygenated in the quartz titration cell by gently passing water-saturated argon over the surface of the enzyme, with stirring, for at least 20 min. After degassing the CcP solution, buffer, EDTA, and riboflavin were added to the cell to give a final solution with 10 mM EDTA, 30 M riboflavin and ~3 M CcP. These solutions were added in the dark and all further experimentation was performed in the dark. After initial absorption measurements were made, the cell was exposed to a 30 W fluorescent light source for a small increment of time, typically 30 to 180 s. The solution was then allowed to equilibrate in the dark, with stirring, for 3 min before the absorbance was measured. Additional exposures to light were made, followed by equilibration and absorbance measurements until greater than 80% of the CcP was reduced.

The equilibrium constant between riboflavin and CcP at each point in the titration was calculated from absorption spectrum and the extinction coefficients of the oxidized and reduced forms of each species. Equilibrium constants determined from data in which both riboflavin and CcP were between 20 and 80% reduced were averaged for each titration. Once the equilibrium constant is determined, the reduction potential of the CcP can be calculated based on the reduction potential of riboflavin. Absorbances at 410 and 466 nm were generally used to determine the concentration of the equilibrated species and the heme iron reduction potentials are based on a value of -0.199 V for the reduction potential of riboflavin at pH 7.0 [23]. If the reduction potential of a CcP mutant differed substantially from that of riboflavin, other redox mediators including safranin O ($E^{\circ'} -0.147$ V) and indigo carmine ($E^{\circ'} -0.116$ V) were used [26].

Results and Discussion

Location of CcP Mutants

Fig. 1 shows the heme environment in CcP [16,17]. 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-175 is replaced by a non-polar amino acid residue [27]. His-175 is coordinated to the heme iron and defines the proximal heme pocket. Trp-191 and Asp-235 are also critical residues in the proximal heme pocket, with Asp-235 hydrogen bonded to both His-175 and Trp-191. Fourteen of the twenty-two CcP mutants used in this study have mutations in the distal heme pocket, two have mutations in the proximal heme pocket and seven have mutations on the surface of CcP.

Fig. 2 shows the location of the surface mutations used in this study. Fig. 2A shows the front face of CcP where Asp-34, Glu-290, and Ala-193 (shown in black) define the high-affinity yeast cytochrome *c* binding site. Pelletier and Kraut have shown that Asp-34 and Glu-290 can potentially form charge-mediated hydrogen bonds with Lys-87 and Lys-73 on yeast cytochrome *c*, respectively, and that both Ala-193 and Ala-194 interact with the solvent-exposed heme edge of yeast cytochrome *c* [28]. Asp-34, Glu-290, and Ala-193 mutants were used in this study. Four other positions on the surface of CcP were also mutated and these include the positions defined by Glu-32, Asp-146, Asp-148, and Glu-291, shown in Fig. 2. Glu-32 and Glu-291 are on the periphery of the high-affinity cytochrome *c* binding site, Fig. 2A, while Asp-146 and Asp-148 are near the entry of the heme access channel, located about 120 °C from the cytochrome *c* binding site, Fig. 2B.

Spectroscopic Properties of CcP Mutants

In order to use the photochemical reduction technique to determine the redox potential of CcP and its mutants, the spectroscopic properties of both the ferric and ferrous forms of the enzyme must be known. While the ferric heme group in CcP is predominantly five-coordinate and high-spin (5c-hs), some of the CcP mutants have spectra that suggest six-coordinate high-spin (6c-hs) or six-coordinate low-spin (6c-ls) heme groups, Fig. 3. Many of the CcP mutants appear to be mixtures of the 5c-hs, 6c-hs, and 6c-ls forms [19,20,27,29–37]. The spectroscopic properties of all the ferric state of CcP and the mutants used in this study are collected in Table 1.

The electronic absorption spectra of ferrous heme proteins are of two basic types, one is characteristic of a five-coordinate high-spin (5c-hs) heme and the other is characteristic of a six-coordinate low-spin (6c-ls) heme. The spectra of ferrous CcP and the 22 mutants used in this study could be described as resulting from 5c-hs and 6c-ls hemes as well as equilibrium mixtures of the two coordination/spin-state forms. Fig. 4 illustrates these three characteristic

ferrous heme spectra using CcP(yst) and two CcP mutants. The peak positions and extinction coefficients for the ferrous forms of all mutants used in this study are collected in Table 2.

The spectrum of wild-type ferrous CcP varies with pH, going from a 5c-hs form at low pH to a 6c-ls form at high pH [38]. 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 [38]. His-52 is thought to coordinate to the heme iron in the high pH form and deprotonation of His-181 is involved in the cooperative ionization [38,39]. The spectrum of wild-type ferrous CcP at pH 7.0 is shown in Fig. 4. At pH 7.0, 96% of wild-type ferrous CcP is five-coordinate and the spectrum shown in Fig. 4 is typical of a 5c-hs ferrous heme protein with α , β , and Soret (γ) bands occurring at 588, 558, and 438 nm, respectively, Table 2. The α band appears as a shoulder on the β band with the extinction coefficient of the α band about half that of the β band.

The spectrum of ferrous CcP(W51H) at pH 7.0 is shown in Fig. 4. This spectrum is typical of a 6c-ls ferrous heme protein and the principle band positions are designated α' , β' , and γ' to distinguish the 6c-ls spectrum from the 5c-hs spectrum. The principle bands for the 6c-ls heme occur at shorter wavelengths compared to 5c-hs form. The α' , β' , and γ' bands for CcP(W51H) occur at 558, 528, and 426 nm, respectively, Table 2. Another characteristic of the 6c-ls form is that the extinction coefficient of the α band is larger than that of the β band.

Most of the CcP mutants used in this study appear to be mixtures of the 5c-hs and 6c-ls forms at pH 7.0, suggesting that the pH-dependent transition has shifted to lower pH in the mutants. A typical mixed-spin state spectrum is shown in Fig. 4 for CcP(D146N). The visible region of the spectrum has a peak at 558 nm with distinct shoulders on both sides of the main peak. The longer wavelength shoulder (588 nm) is the α band of the 5c-hs form and the shorter wavelength shoulder (528 nm) is the β' band of the 6c-ls form. Both the α' band of the 6c-ls form and the β band of the 5c-hs form contribute to the absorption maximum at 558 nm. The Soret band is asymmetric with clear contributions from both five- and six-coordinate forms and these are indicated by γ and γ' , respectively, Fig. 4. The Soret maximum occurs at 428 nm in this mutant, characteristic of the 6c-ls form, but there is a distinct shoulder at 440 nm, characteristic of the 5c-hs form.

Inspection of Table 2 indicates that the maximum absorbance in the visible region of the spectrum occurs at 558 ± 2 nm for CcP and all of the CcP mutants except CcP(H52Q). Ferrous CcP(H52Q) appears to be a predominantly five-coordinate species at pH 7.0 with β band occurring at 552 nm compared to 558 nm for wild-type CcP. The Soret band in ferrous CcP(H52Q) also has an unusual shoulder at 418 nm that is attributed to a small amount of six-coordinate form, Table 2. Bateman *et al.* [40] have shown that CcP(H52Q) does not react with hydrogen peroxide to form the oxidized enzyme intermediate called Compound I but that CcP(H52Q) catalyzes the oxidation of hydrogen peroxide to molecular oxygen in an electrochemical process. Apparently, the unusual properties of CcP(H52Q) are also reflected in the spectrum of the ferrous enzyme.

Another important observation related to the spectra of the ferrous forms concerns the His-52 mutants. Most of the ferrous His-52 mutants have spectral contributions attributed to six-coordinate forms indicating that His-52 is not the only potential heme ligand in the ferrous enzyme. Ferrous CcP(H52L) is a mixed-spin species and the most likely candidate for the sixth heme ligand is a hydroxide ion.

Fe(III) Reduction Potentials of CcP and CcP Mutants at pH 7.0

The reduction potentials of wild-type baker's yeast CcP, three cloned forms of CcP, and 26 CcP mutants are collected in Table 3. The reduction potentials for 22 of the mutants were determined in this study and four reduction potentials are previously published values. The

reduction potential of baker's yeast CcP at pH 7.0, 25 °C was previously determined to be -194 ± 3 mV by potentiometric methods [38]. The photochemical reduction technique used in this study gives a value of -189 ± 3 mV, within the estimated error of the two techniques. The $E^{\circ'}$ value of -189 mV for CcP(yst) found in this study will be used for comparison purposes. The reduction potential of CcP(MI) is essentially identical to that of CcP(yst) and those of CcP (MKT1) and CcP(MKT2) are only slightly more positive than the reduction potential of baker's yeast CcP, Table 2 [25,41,42].

The CcP mutants used in this study are divided into three groups, heme distal pocket mutants, proximal pocket mutants and surface mutants. It was expected that the heme pocket mutants would have larger effects on the observed reduction potentials than the surface mutants and this proved to be the case. For the heme pockets mutants, the $E^{\circ'}$ values range from -259 mV for CcP(H52N) to -78 mV for CcP(D235A). The surface mutants were clustered in two areas, Fig. 2. The first area is within the cytochrome *c* binding site [28] and the second area is near the heme access channel [17]. Within both of these areas, the mutations tend to be charge-neutralization mutants, *i.e.*, the replacement of a negatively charged amino acid residue in the wild-type enzyme to a neutral amino acid in the mutant. The smaller negative charge of the surface of CcP in the charge-neutralization mutants should destabilize the Fe(III) state in the mutant relative to the wild-type enzyme and we would expect a more positive reduction potential in the mutant. This was observed with the surface mutants having reduction potentials between -162 and -177 mV, 12 to 27 mV more positive than that of the wild-type enzyme, Table 3.

Distal Pocket CcP Mutants

Three residues form the distal heme pocket in CcP, Arg-48, Trp-51, and His-52, Fig. 1. Fourteen distal pocket mutants have been constructed and of this group, four mutants have negatively shifted reduction potentials compared to the wild-type enzyme and ten have positively shifted reduction potentials. In most cases the effect of distal pocket mutations can be understood in terms of electrostatic destabilization of the ferric state relative to the ferrous state. The Fe(III) state of the heme has a net positive charge delocalized within the iron-porphyrin system relative to the Fe(II) state.

Position 48 Variants

The reduction potentials of two position 48 variants were determined, CcP(R48K) and CcP(R48L). The more conservative variant replaces the positively charged arginine residue with a potentially positively charged lysine residue and its reduction potential is within experimental error of that of the wild-type enzyme. Replacing the arginine residue with a nonpolar leucine group increases $E^{\circ'}$ by 25 mV, consistent with destabilization of the Fe(III) heme in a more nonpolar environment.

Bujons *et al.* [42] have made a non-conservative mutation at position 48, replacing the positively charged arginine residue with a negatively charged glutamate. They were unable to measure $E^{\circ'}$ at pH 7 for the mutant but did obtain a value of -179 mV at pH 6.0, Table 3. This is a shift of -50 mV relative to the $E^{\circ'}$ value of -129 mV obtained for wild-type CcP(MKT2) at pH 6.0 suggesting that the negatively charged glutamate can stabilize the positively charged Fe(III) heme better than Arg-48 although the change is perhaps smaller than expected. Bujons *et al.* suggest that changes in heme ligation may compensate for the purely electrostatic effect of adding a nearby negative charge [42].

Position 51 Variants

Only one point mutation at position 51 was investigated, CcP(W51H). His-51 is most likely unprotonated and this mutation replaces one polar uncharged residue with another. The effect

of the mutation is small, with $E^{\circ'}$ becoming more negative by 11 mV compared to $E^{\circ'}$ for CcP (yst), Table 3. CcP(W51H) has interesting spectroscopic properties. In the Fe(III) state, the spectrum of CcP(W51H) is that of a pure 6c-hs form at pH 6.0 with one of the highest extinction coefficients we have measured among CcP mutants, $159 \text{ mM}^{-1} \text{ cm}^{-1}$ at the Soret maximum, Fig. 3 and Table 1 [30]. Increasing the pH to 8.0 causes almost complete conversion to a 6c-ls form [30]. The CcP(W51H) is also the only mutant investigated that has a pure 6c-ls ferrous heme spectrum at pH 7.0, Fig. 4.

CcP variants with multiple point mutations including changes at position 51 were also constructed. The properties of these CcP variants will be discussed below.

Position 52 Variants

In many respects, His-52 is the key residue in promoting the reaction between hydrogen peroxide and CcP. Replacing His-52 with the nonpolar leucine residue decreases the rate of the hydrogen peroxide reaction by about five orders of magnitude [27]. On the other hand, the H52L mutation has only a small effect on $E^{\circ'}$, causing the reduction potential to become more positive by 19 mV, consistent with increasing nonpolarity of the heme pocket and destabilization of the Fe(III) heme.

The most conservative changes at position 52 are those that replace the neutral but polar histidine side chain with other uncharged polar residues such as asparagine and glutamine. Both CcP(H52N) and CcP(H52Q) have more negative redox potentials than wild-type CcP, indicating that the Fe(III) state is stabilized in these two mutations compared to wild-type enzyme. The observed changes are relatively large, -70 mV for CcP(H52N) and -35 mV for CcP(H52Q) (Table 3). In fact, CcP(H52N) has the most negative redox potential of all CcP variants examined in this study, with $E^{\circ'}$ equal to -259 mV .

Somewhat surprisingly, the seemingly more drastic variations in the heme pocket, replacing His-52 with either an aspartate and glutamate residue have smaller effects than replacement of His-52 with asparagine and glutamine. It was expected that both the aspartate and glutamate side chains would be deprotonated in the distal heme pocket, producing a negatively charged residue near the heme iron causing large decreases in the reduction potential. This did not happen. $E^{\circ'}$ for CcP(H52E) is actually more positive by 6 mV than CcP(yst). It is possible, but unlikely, that the aspartate and glutamate residues remain protonated in the heme pocket, producing smaller than expected changes in the reduction potential. It is also possible that the hydrogen-bonding pattern in the distal heme pocket is severely altered with the aspartate and glutamate residues interacting with Arg-48. An Asp-52/Arg-48 or Glu-52/Arg-48 interaction would neutralize the charge on both residues, substantially decreasing the electrostatic field in the distal heme pocket and producing a small effect on the redox potential.

Replacing His-52 with a lysine residue puts a potentially positive charge close to the heme iron. $E^{\circ'}$ for CcP(H52K) is 32 mV more positive than $E^{\circ'}$ for CcP(yst). The expected destabilization of the Fe(III) state occurs, but the effect is not as large as might be expected. The pK_a for the ϵ -amino group of Lys-52 in CcP(H52K) appears to be 6.2 in the ferric state of the enzyme, causing this residue to be largely unprotonated at pH 7.0 [34].

CcP(W51H/H52L)

During the course of these studies, a double mutation altering the position of the distal histidine was constructed in order to investigate the effect of histidine location on the reaction between hydrogen peroxide and the enzyme [30]. The reactivity of CcP(W51H/H52L) with hydrogen peroxide is about four-orders of magnitude slower than that of wild-type CcP [30]. $E^{\circ'}$ for the double mutant is 27 mV more positive than that of CcP(yst) due to the more nonpolar character

of the heme pocket. An interesting observation is that E° for CcP(W51H) is more negative than the E° for wild-type CcP(yst) and E° for CcP(H52L) is more positive than that of wild type enzyme, but the double mutation, CcP(W51H/H52L), results in a more positive shift than the CcP(H52L) alone. The result of the double mutation is not a simple additive function of the single mutations.

Triple Mutations

The final set of distal pocket mutations involved all three distal pocket residues. The goal was to make the distal pocket very nonpolar by converting Arg-48, Trp-51 and His-52 to nonpolar residues simultaneously. The three CcP variants had all alanine, all valine, or all leucine residues at positions 48, 51, and 52. All three of these mutants resulted in more positive values of E° compared to wild-type CcP with the changes correlating with the size of the apolar side chain. E° values for the triple alanine, triple valine, and triple leucine mutants were 26, 39, and 43 mV more positive than E° for CcP(yst), respectively. The triple leucine mutant had the most positive redox potential of any CcP variant investigated in this study with an E° value of -146 mV at pH 7.0, although there are reports of more positive values in the literature [21,41]. The most positive values of the reduction potential occur with proximal heme pocket mutations.

Proximal Pocket CcP Mutants

Important residues on the proximal side of the heme include the proximal heme ligand, His-175, Trp-191, which is the site of the free radical in CcP Compound I, and Asp-235, which hydrogen bonds to both His-175 and Trp-191 and is thought to donate electron density to the heme via His-175, Fig. 1. The effect of His-175, Trp-191, and Asp-235 mutations have been investigated by previous workers [21,25,41]. In this study we re-determined the reduction potential of CcP (W191F) and also have investigated the double mutant CcP (H52L/W191F).

Position 175

Choudhury *et al.* [21] constructed four proximal pocket mutants, H175C, H175E, H175Q and a double mutant H175Q/W191F using CcP(eco) as the parental clone. All four mutants retained catalytic activity although diminished relative to wild-type CcP. All four mutants were unstable at alkaline pH and reduction potentials determinations were made at pH 5.5. CcP(eco) has an E° (pH 5.5) of -102 mV, similar to that of wild-type yeast CcP previously determined to be -109 mV by potentiometry [21,38] and -99 mV by the photochemical titration techniques used in this study. The only His-175 mutant stable enough for a reduction potential measurement was that of H175Q with an estimated value of -3 mV at pH 5.5 (Fig. 5b in [21]), about 100 mV more positive than the parental CcP(eco). There is a large destabilization of the Fe(III) state caused by replacing the imidazole side chain of His-175 with the amide group of Gln-175. Interpretation of the electron density in the crystal structure of the H175Q mutant suggests that the carbonyl oxygen of the side chain amide group is coordinated to the heme iron while the amide nitrogen group hydrogen bonds to Asp-235 [21]. These results are consistent with the idea that the imidazole side chain can more readily transfer negative charge from Asp-235 into the heme iron than the amide group of Gln-175. The increased electron donation into the heme iron stabilizes the higher redox states of the heme iron, especially the Fe(IV) site in CcP compound I [17].

Position 191

Two mutations, a single CcP(W191F) variant and a double CcP(H52L/W191F) mutation, were examined in this study. A CcP(MI,W191F) variant has been previously investigated by Mauro *et al.* [25]. Replacement of Trp-191 with phenylalanine is not expected to greatly change the reduction potential. Mauro *et al.* found an E° value of -202 mV for CcP(MI,W191F). In this

study, the $E^{\circ'}$ value for the W191F mutant derived from CcP(eco) was -187 mV at pH 7.0, somewhat lower than the Mauro *et al.* value. Part of this difference can be attributed to different reduction potentials assigned to riboflavin in the two studies. Mauro *et al.* used a value of -205 mV for the riboflavin reduction potential at pH 7.0 while a value of -199 mV was used in this study [23].

Individually the W191F mutation has no effect on the reduction potential and the H52L mutation caused a 19 mV positive shift in $E^{\circ'}$. The CcP(H52L/W191F) mutation was expected to be similar to CcP(H52L). Surprisingly, the double mutation resulted in a significantly more positive value for $E^{\circ'}$, a value of -151 mV, 38 mV more positive than the $E^{\circ'}$ value of CcP(yst) and 19 mV more positive than CcP(H52L), again demonstrating that the changes in the reduction potential of multiple site variants are not additive functions of the individual point mutations.

Position 235

While not investigated in this study, the results of an investigation into the properties of a number of Asp-235 mutants by Goodin and McRee [41] are an important component in the interpretation of the shifts in reduction potentials in the CcP mutants. Goodin and McRee examined three mutations at position 235, D235E, D235N, and D235A using CcP(MKT1) as the parental clone. It was expected that the removal of the native aspartate acid side chain would result in not only destabilization of the Fe(III) state from electrostatic considerations but also from the influence of this group on His-175 and Trp-191, both of which contribute to the Fe(III) stability. The most conservative change is replacing the aspartate side chain with glutamate. Crystal structures obtained by Goodin and McRee indicated that the glutamate was in position to hydrogen bond with both Trp-191 and His-175. The geometry of these bonds was altered, resulting in non-ideal bond angles. While it is theorized that the histidine in native CcP is largely deprotonated, with an imidazolate side chain, the new bond angle in the D235E mutant results in a much stronger probability that the imidazole side chain of His-175 is in the neutral form. Changing the His-175 side chain from an imidazolate group to a neutral imidazole should cause the reduction potential of the D235E mutant to become more positive. The reduction potential of D235E does indeed become more positive, shifting to a value of -113 mV, 70 mV more positive than CcP(yst).

The effects of the remaining mutations D235N and D235A were initially difficult to explain. Both variants were expected to result in a more positive reduction potential as the hydrogen bond to His-175 and the possible negative charge would be lost. The D235N mutation resulted in an $E^{\circ'}$ of -79 mV and the D235A mutation resulted in an $E^{\circ'}$ of -78 mV, 104 and 105 mV more positive than CcP(MKT1), the largest positive shifts in the reduction potential on any CcP mutants yet investigated. Although both mutants resulted in the expected positive shift of reduction potential, the difference between the shifts was only 1 mV. The D235A mutation where the possibly negative hydrogen bonding aspartate is replaced by an uncharged, nonpolar residue incapable of producing hydrogen bonds was expected to have a much greater shift than the D235N, which retains a polarity but not the possible charge. Zhou [43] explained the small difference in reduction potential between the D235A and D235N mutants by modeling of the mutations. The amide dipole of Asn-235 was found to be at an $\sim 100^\circ$ angle from the vector to the heme iron, resulting in an almost complete cancellation of the effects of the asparagine polarity. This loss of effective polarity effectively explains the very small difference between the effects of substituting alanine or asparagine for the native aspartate in the 235 position.

Surface Mutants

Two different types of surface mutants were modified. The first set was the residues near the hydrogen peroxide access channel in CcP, D146N and D148N, Fig. 2B [17]. The second set

of mutations involved changing charged residues on the surface of CcP that are thought to involve the cytochrome c binding site, Fig. 2A [28]. In all but one case the mutation was to change a negatively charged carboxylate group to a neutral amide group. The last mutation was to change a small aliphatic to a large aromatic residue CcP(A193F). From the theory of Kassner [4,5], it was expected that the $E^{\circ'}$ for all of these mutants would be more positive than native CcP and this is what was observed.

The five point mutations involving carboxylate to amide substitutions were located from 11.6 to 27.2 Å from the heme iron and all resulted in positive shifts in the potential. There did not appear to be a correlation between the distance from the heme iron and the magnitude of the positive shift, most likely because the surface mutations caused changes in heme ligation in the two redox states. In order of increasing distance from the heme iron, the following shifts were observed: D146N (11.6 Å, +16 mV), D34N (23.7 Å, +14 mV), E291Q (26.2 Å, +27 mV), E290N (26.4 Å, +12 mV), and E32Q (27.2 Å, +21 mV). Asp-148 is located within 14.4 Å of the heme iron and it was expected that the double mutant D146N/D148N would have a larger positive shift than any of the single point mutants. However, this was not the case. The reduction potentials of the single mutant, D146N, and the double mutant, D146N/D148N were the same, Table 3.

Rodgers and Sligar [44] have determined the effect of surface charge-neutralization and charge-reversal mutations on the reduction potential of rat liver cytochrome b_5 and find shifts of from -6 to + 20 mV relative to the wild-type protein, about the same magnitude as observed with CcP. The maximum negative shift of -6 mV was due to a mutation that introduced a negative charge on the surface close to the solvent exposed heme edge in cytochrome b_5 .

Conclusions

As previously stated, the research goals of this research project were threefold. The first goal was to investigate the effects of distal pocket mutations on the Fe(III)/Fe(II) redox couple in cytochrome c peroxidase. Within the heme pocket, $E^{\circ'}$ (pH 7) was determined for fifteen CcP variants including ten single mutants, two double mutants, and three triple mutants, Table 3. It was found that the reduction potential shifts for most cases could be explained by electrostatic interactions with the mutations stabilizing or destabilizing the Fe(III) heme in the mutant relative to the Fe(III) heme in wild-type CcP. For those cases where the mutation did not result in the predicted shift in direction or magnitude of the reduction potential, structural effects that negated the expected electrostatic results were proposed, as in the case of H52D and H52E. A most interesting observation is that the neutral H52N and H52Q mutations caused larger potential shifts than did mutations that introduce a negative charge into the distal heme pocket, H52D and H52E. Ion-pairing with Arg-48, alterations in the hydrogen bonding networks, and changes in the heme ligation were proposed to explain the smaller effects of H52D and H52E relative to H52N and H52Q. Obtaining crystal structures of these four distal pocket mutants would make an important contribution in understanding the changes in redox potential.

Understanding the effects of surface mutations on the Fe(III) reduction potential was the second goal of this study. In general, the surface mutations used in this study increased the non-polar surface area on the mutant relative to that in wild-type CcP and this was expected to cause positive shifts in the reduction potential. It was also expected that surface mutations would have smaller effects than heme pocket mutations. Both expectations were met but the shifts could not be rationalized based strictly on electrostatic effects, *i.e.*, that neutralization of a negatively-charged residue closer to the heme iron would cause a larger shift than neutralization of a more distant charged residue. This is most likely due to the plasticity of heme ligation in CcP and the observation that surface mutations caused changes in heme ligation in both the ferric and ferrous forms of the CcP mutants compared to wild-type enzyme. Changes in heme

ligation would be expected to have larger effects on the reduction potential than the neutralization of a distant changed residue.

Finally, using the entire range of CcP mutants investigated to date, it appears as if the Fe(III) reduction potential can be varied by about +100 mV [21,41] in the positive direction and -70 mV (this study) in the negative direction. Mutants at both ends of the E^0 scale retain reactivity toward hydrogen peroxide and should be investigated for their effects on the stability of CcP compound I, susceptibility to heme degradation in the presence of hydrogen peroxide, and potential peroxxygenase activity.

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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 *c* peroxidase

CcP(yst)

wild-type CcP isolated from baker's yeast

CcP(eco)

cloned recombinant CcP expressed in *E. coli* with the exact amino acid sequence as CcP(yst)

CcP(MI)

cloned recombinant CcP expressed in *E. coli* that differs from CcP(yst) by having a Met-Leu dipeptide fused to the N-terminus and two point mutations, T53I and D152G

CcP(MKT1)

cloned recombinant CcP expressed in *E. coli* which differs from CcP(yst) at the following positions T1M, T2K, P3T, T53I, and D152G

CcP(MKT2)

cloned recombinant CcP which differs from CcP(yst) at the first three positions, T1M, T2K, and P3T

E°'

standard reduction potential at pH 7.0

Fig 1.

Heme environment in CcP(yst) [17]. 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.

Fig 2.

Panel A shows a space-filling model of the front face of CcP(yst). Asp-34, Ala-193, and Glu-290 (shown in black) define the high-affinity yeast cytochrome *c* binding site [28]. Glu-32 and Glu-290 (shown in blue) are sites of mutation used in this study. Panel B shows CcP rotated 120° counter-clockwise about a vertical axis relative to the molecule shown in Panel A. This view shows the heme access channel [17] and the buried heme group (shown in red). Asp-146 and Asp-148 (shown in blue) are near the entrance to the heme access channel. See the online issue of this article for a color version of this figure.

Fig 3.

Spectra of the Fe(III) redox state of CcP(yst) and two CcP mutants. These three spectra represent the three principle coordination/spin state combinations observed in ferric heme proteins. Thin solid line - CcP(yst) at pH 6.0. The spectrum of CcP(yst) is an example of a predominantly five-coordinate, high-spin (5c-hs) heme protein. Dashed line - CcP(W51H) at pH 6.0 is an example of a predominantly six coordinate, high-spin (6c-hs) heme protein. Water is the most probable sixth ligand in CcP(W51H). Thick solid line - CcP(D235N) at pH 7.5 is an example of a predominantly six-coordinate, low-spin (6c-ls) heme protein. His-52 is the most likely sixth ligand in CcP(D235N) at pH 7.5 [37].

Fig 4.

Spectra Fe(II) CcP and two Fe(II) CcP mutants at pH 7.0. Thin solid line - The spectrum of CcP(yst) is characteristic of a predominantly five-coordinate, high-spin heme (5c-hs) with major band positions indicated by α , β , and γ (Soret). Dashed line - The spectrum of CcP (W51H), with band positions indicated by α' , β' , and γ' (Soret), is characteristic of a heme group that is predominantly six-coordinate and low-spin (6c-ls). Thick solid line - The spectrum of CcP(D146N) indicates that this mutant is a mixture of 5c-hs and 6c-ls heme species with significant absorption at the α , α' , β , β' , γ and γ' positions. Note especially the asymmetric Soret band. The sodium hydrosulfite used to reduced CcP and the two mutants obscures the protein spectrum below 400 nm.

Table 1
Spectroscopic properties of ferric cytochrome *c* peroxidase and some cytochrome *c* peroxidase mutants.^a

Mutation	clone	pH	Protein	δ	Soret	CT1 ^c	β	α	CT2 ^c	Ref ^b
Native Proteins										
CcP	yst	6.0	282 (76)	380 (64) sh	408 (98)	508 (11.3)	544 (8.6) sh	590 (3.3) sh	647 (3.4)	19,29
CcP	MI	6.0	280 (78)	380 (66) sh	408 (102)	508 (11.4)	540 (9.5) sh	592 (3.3) sh	644 (3.4)	20,29
CcP	eco	6.0	282 (76)	380 (66) sh	408 (101)	508 (11.4)	540 (9.6) sh	592 (3.6) sh	644 (3.6)	30,TW
Disal Pocket Mutants										
R48K	MI	6.0	282 (81)	382 (65) sh	411 (105)	520 (11.2)	542 (10.1) sh	-	648 (2.7)	31,32
R48L	MI	6.0	282 (82)	378 (76) sh	405 (97)	510 (12.4)	542 (9.6) sh	594 (2.9) sh	648 (3.7)	31,32
R48A/W51A/	eco	6.0	280 (75)	371 (52) sh	405 (92)	508 (9.2)	540 (8.1) sh	588 (4.8) sh	636 (3.6)	33
H52A										
R48V/W51V/	eco	6.0	278 (77)	378 (75) sh	406 (93)	515 (11.2)	550 (7.2) sh	590 (4.4) sh	640 (3.3)	33
H52V										
R48L/W51L/	eco	6.0	282 (103)	380 (98) sh	401 (117)	505 (16.2)	535 (13) sh	580 (6.8) sh	673 (5.8)	33
H52L										
W51H	eco	6.0	281 (76)	365 (34) sh	409 (159)	505 (9.8)	541 (7.1) sh	583 (4.2) sh	633 (4.8)	30
W51H/H52L	eco	6.0	281 (76)	362 (34) sh	408 (160)	505 (10.3)	539 (7.0) sh	584 (3.8) sh	634 (4.6)	30
W51H/	eco	6.0	278 (90)	359 (32) sh	409 (137)	-	529 (9.5)	566 (6.5) sh	632 (3.1)	30
H52W										
H52D	eco	6.0	282 (87)	378 (67) sh	407 (118)	504 (11.8)	537 (9.3) sh	583 (3.9) sh	635 (4.1)	30
H52E	eco	6.0	282 (84)	380 (76) sh	406 (99)	507 (12.0)	540 (9.6) sh	593 (3.4) sh	647 (3.9)	30
H52K	eco	6.0	282 (79)	382 (67) sh	408 (87)	512 (10.8)	540 (9.4) sh	584 (4.0) sh	649 (3.1)	30,34
H52L	MI	6.0	282 (84)	379 (81) sh	404 (97)	511 (12.7)	544 (9.8) sh	595 (3.0) sh	651 (4.0)	27,35
H52L	eco	6.0	282 (82)	383 (82) sh	404 (97)	511 (12.7)	542 (10.1) sh	596 (3.3) sh	651 (4.0)	33,36
H52N	eco	6.0	282 (86)	382 (76) sh	405 (99)	507 (12.9)	546 (9.5) sh	597 (3.0) sh	647 (3.8)	30
H52Q	eco	6.0	282 (81)	382 (72) sh	407 (105)	508 (11.9)	541 (9.2) sh	596 (2.9) sh	642 (3.7)	30
Proximal Pocket Mutants										
W191F	MI	7.0	278 (89)	378 (62) sh	408 (109)	506 (10.8)	544 (9.7) sh	592 (3.8) sh	644 (2.9)	29,31
W191F/	eco	7.0	280 (68)	380 (66) sh	406 (89)	508 (9.8)	542 (7.8) sh	590 (2.9) sh	642 (2.9)	TW
H52L										
D235N	MI	7.5	280 (75)	358 (28) sh	411 (111)	-	533 (10.3)	561 (8.6) sh	-	31,37
Surface Mutants										
D146N	eco	7.0	282 (78)	381 (72) sh	408 (100)	510 (11.9)	537 (9.7) sh	594 (3.3) sh	642 (3.6)	TW
D146N/	eco	7.0	282 (84)	380 (76) sh	408 (109)	508 (12.2)	542 (9.9) sh	588 (3.9) sh	642 (3.5)	TW
D148N										
E32Q	MI	7.0	282 (86)	380 (67) sh	408 (109)	510 (11.5)	542 (9.6) sh	588 (3.4) sh	644 (3.1)	TW
D34N	MI	7.0	282 (89)	376 (66) sh	408 (113)	510 (12.0)	544 (9.6) sh	588 (3.5) sh	644 (3.2)	TW
A193F	MI	7.0	282 (83)	380 (63) sh	408 (104)	512 (10.8)	538 (9.6) sh	598 (2.8) sh	648 (3.1)	TW
E290N	MI	7.0	282 (85)	376 (65) sh	408 (110)	510 (11.7)	542 (9.5) sh	592 (2.1) sh	648 (3.1)	TW

^a Data entries include both the wavelength (λ) and extinction coefficient (ϵ) of maxima and prominent shoulders in the format ' $\lambda(\epsilon)$ ' with λ given in nm and ϵ given in $\text{mM}^{-1}\text{cm}^{-1}$. Shoulders are designated by *sh*.

^b *TW* indicates this work.

^c CT1 and CT2 represent the charge transfer bands in the high-spin derivatives.

Table 2

Spectroscopic parameters of ferrous CcP and ferrous CcP mutants at pH 7.0.^a

Mutant	Clone	Soret (γ')	Soret (γ)	β'	α'/β	α
CcP	Eco	-	438 (95)	-	558 (14)	588 (7.7) <i>sh</i>
Distal Pocket Mutants						
R48K	MI	428 (111)	440 (84) <i>sh</i>	528 (9.2) <i>sh</i>	560 (15)	590 (5.7) <i>sh</i>
R48L	MI	430 (126)	436 (121) <i>sh</i>	530 (11) <i>sh</i>	560 (18)	592 (6.6) <i>sh</i>
R48A/W51A/H52A	Eco	430 (105)	-	530 (8.6) <i>sh</i>	556 (12)	-
R48V/W51V/H52V	Eco	430 (113)	-	530 (8.8) <i>sh</i>	558 (13)	-
R48L/W51L/H52L	Eco	426 (124)	432 (115) <i>sh</i>	526 (9.7) <i>sh</i>	556 (14)	590 (5.8) <i>sh</i>
W51H	Eco	426 (123)	-	528 (10)	558 (17)	-
W51H/H52L	Eco	428 (106)	-	530 (9.8) <i>sh</i>	558 (14)	588 (5.8) <i>sh</i>
H52D	Eco	426 (92)	-s	530 (8.2) <i>sh</i>	556 (12)	586 (5.2) <i>sh</i>
H52E	Eco	424 (82)	434 (75) <i>sh</i>	528 (7.0) <i>sh</i>	56 (9.9)	585 (4.6) <i>sh</i>
H52N	Eco	-	440 (104)	529 (6.5) <i>sh</i>	560 (13)	594 (6.1) <i>sh</i>
H52Q	Eco	418 (68) <i>sh</i>	432 (82)	-	552 (9.4)	586 (4.5) <i>sh</i>
H52K	Eco	-	434 (84)	-	558 (10)	592 (5.1) <i>sh</i>
H52L	Eco	424 (77) <i>sh</i>	434 (85)	526 (7.0) <i>sh</i>	558 (11)	594 (5.1) <i>sh</i>
Proximal Pocket Mutants						
H52L/W191F	Eco	-	434 (92)	-	558 (11)	592 (5.1) <i>sh</i>
W191F	MI	430 (94)	438 (88) <i>sh</i>	530 (7.8) <i>sh</i>	560 (13)	592 (5.5) <i>sh</i>
Surface Mutants						
E32Q	MI	428 (93) <i>sh</i>	434 (94)	526 (8.0) <i>sh</i>	558 (14)	592 (6.5) <i>sh</i>
D34N	MI	430 (97)	438 (92) <i>sh</i>	532 (8.8) <i>sh</i>	558 (14)	588 (6.8) <i>sh</i>
D146N	Eco	428 (92)	440 (82) <i>sh</i>	528 (8.9) <i>sh</i>	558 (14)	588 (7.3) <i>sh</i>
D146N/D148N	Eco	428 (88)	438 (81) <i>sh</i>	539 (8.2) <i>sh</i>	558 (13)	592 (6.0) <i>sh</i>
A193F	MI	428 (95)	440 (78) <i>sh</i>	530 (8.4) <i>sh</i>	560 (14)	592 (5.9) <i>sh</i>
E290N	MI	430 (96)	438 (86) <i>sh</i>	532 (8.2) <i>sh</i>	558 (13)	592 (5.9) <i>sh</i>
E291Q	MI	428 (104) <i>sh</i>	436 (105)	530 (8.0) <i>sh</i>	558 (14)	590 (6.3) <i>sh</i>

^aData entries include both the wavelength (λ) and extinction coefficient (ϵ) of maxima and prominent shoulders in the format ' $\lambda(\epsilon)$ ' with λ given in nm and ϵ given in $mM^{-1} cm^{-1}$. Shoulders are designated by *sh*. Absorption bands attributed to five-coordinate, high-spin ferrous derivatives are designated as the α , β , and Soret (γ) bands. Absorption bands attributed to six-coordinate, low-spin derivatives are designated as the α' , β' , and Soret (γ') bands.

Table 3
Reduction Potentials of Yeast CcP, Recombinant CcP, and CcP Mutants at pH 7.0, 25°C.

Mutation	Clone	E ^{0'} (mV)	Mutation	Clone	E ^{0'} (mV)
Wild-Type Enzyme			H52K	eco	-157
CcP	yst	-189, -194 ^a	H52L	eco	-170
CcP	MI	-194 ^b	Proximal Pocket Mutants		
CcP	MKT1	-183 ^c	H52L/W191F	eco	-151
CcP	MKT2	-186 ^d	W191F	MI	-202 ^b
Distal Pocket Mutants			W191F	eco	-187
R48K	MI	-186	D235A	MKT1	-78 ^c
R48L	MI	-164	D235E	MKT1	-113 ^c
R48E	MKT2	-179 ^d	D235N	MKT1	-79 ^c
R48A/W51A/H52A	eco	-163	Surface Mutants		
R48V/W51V/H52V	eco	-150	E32Q	MI	-168
R48L/W51L/H52L	eco	-146	D34N	MI	-175
W51H	eco	-200	D146N	eco	-173
W51H/H52L	eco	-162	D146N/D148N	eco	-173
H52D	eco	-221	A193F	MI	-170
H52E	eco	-183	E290N	MI	-177
H52N	eco	-259	E291Q	MI	-162
H52Q	eco	-224			

^aData from reference 38.

^bData from reference 25.

^cData from reference 41.

^dData from reference 42, the E^{0'} values for MKT2 and R48E were determined at pH 7.0 and 6.0, respectively.