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Apolar distal pocket mutants of yeast cytochrome c peroxidase: Binding of imidazole, 1-methylimidazole and 4-nitroimidazole to the triAla, triVal, and triLeu variants

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Abstract

Imidazole binding to three apolar distal heme pocket mutants of yeast cytochrome c peroxidase (CcP) has been investigated between pH 4 and 8. The three CcP variants have Arg-48, Trp-51, and His-52 mutated to either all alanine, CcP(triAla), all valine, CcP(triVal), or all leucine residues, CcP(triLeu). The imidazole binding curves for all three mutants are biphasic indicating that each of the mutants exist in at least two conformational states with different affinities for imidazole. At pH 7, the high-affinity conformations of the three CcP mutants bind imidazole between 3.8 and 4.7 orders of magnitude stronger than that of wild-type CcP while the low-affinity conformations have binding affinities about 2.5 orders of magnitude larger than wild-type CcP. Imidazole binding to the three CcP mutants is pH dependent with the strongest binding observed at high pH. Apparent pK_a values for the transition in binding vary between 5.6 and 7.5 for the high-affinity conformations and between 6.2 and 6.8 for the low-affinity conformations of the CcP triple mutants. The kinetics of imidazole binding are also biphasic. The fast phase of imidazole binding to CcP(triAla) and CcP(triLeu) is linearly dependent on the imidazole concentration while the slow phase is independent of imidazole concentration. Both phases of imidazole binding to CcP(triVal) have a hyperbolic dependence on the imidazole concentration. The apparent association rate constants vary between 30 and 170 $M^{-1}s^{-1}$ while the apparent dissociation rate constants vary between 0.05 and 0.43 s^{-1} . The CcP triple mutants have higher binding affinities for 1-methylimidazole and 4-nitroimidazole than does wild-type CcP.

Keywords

Cytochrome c peroxidase; Distal pocket mutants; Imidazole derivatives; Imidazole binding

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1. Introduction

Heme enzymes are ubiquitous in nature and catalyze a wide variety of oxidation reactions utilizing either molecular oxygen or hydrogen peroxide as oxidant and some heme enzymes have been used in the synthetic laboratory to catalyze specific oxidations [1,2]. With advances in recombinant DNA technology and the ability to modify protein structure through site-directed mutagenesis, significant effort has gone into the development of enzymes for use as catalysts in organic synthesis [3,4]. The utilization of hydrogen peroxide for oxygenation of organic substrates, the so-called “peroxygenase” activity, is a reaction of interest to synthetic chemists since it mimics the monooxygenase reactions of the cytochrome P450s without the need for the additional reductase and cofactor (NADH or NADPH) of the P450 system. Yeast cytochrome *c* peroxidase (CcP) appears to be a good candidate to serve as a scaffold for developing specific peroxygenases for synthetic purposes. CcP has a low native peroxygenase activity, oxidizing styrene to styrene oxide with steady-state turnover rates of about 10^{-4} s^{-1} at pH 7 [5] and it is very stable to oxidative degradation by hydrogen peroxide [6]. Two factors for the low peroxygenase activity of CcP may be due to the low affinity for small organic substrates within the distal heme pocket and lack of accessibility of the substrate to the heme iron for direct oxygen atom transfer during the catalysis. To test these hypotheses, three CcP mutants were constructed to make the distal heme pocket more apolar [7]. The mutants were constructed by simultaneously replacing Arg-48, Trp-51, and His-52 by either all alanines, CcP(triAla), all valines, CcP(triVal), or all leucines, CcP(triLeu). (See Fig. 1 of the preceding paper [8] for the active site structure of CcP). All three mutants show a 30- to 34-fold increase in the rate of 1-methoxynaphthalene hydroxylation by hydrogen peroxide [9]. The hydroxylation activity by the CcP mutants is only a factor of two slower than naphthalene hydroxylation by rat liver microsomal cytochrome P450.

The three apolar CcP mutants have been characterized by UV-vis spectroscopy, catalytic activity, reaction with hydrogen peroxide, and by cyanide binding [7]. The UV-vis spectrum of all three mutants are pH dependent indicating a change in heme ligation over the pH range 4 to 8. The three mutants retain less than 0.02% of wild-type activity, primarily due to the decrease in the rate of reaction with hydrogen peroxide. Binding of cyanide to the three apolar mutants is biphasic indicating that there are at least two slowly-exchanging conformations in each mutant, and that these different conformational states have different cyanide affinities. The binding affinity for cyanide is reduced by at least two orders of magnitude compared to wild-type CcP and the rate of cyanide binding is decreased by at least four orders of magnitude. In this manuscript we report on the further characterization of CcP(triAla), CcP(triVal), and CcP(triLeu) by an investigation of imidazole binding. In contrast to the loss of cyanide affinity, the three apolar mutants show a substantial increase in imidazole affinity compared to wild-type CcP. In the preceding paper, we have shown that wild-type yeast cytochrome *c* peroxidase (yCcP) and a recombinant form of CcP (rCcP) have very weak affinities for imidazole with equilibrium dissociation constants (K_D) of about 4 M at pH 7 [8]. These are the weakest imidazole binding affinities reported for any heme protein. Typically, heme protein-imidazole complexes have K_D values in the millimolar to micromolar region [10–14]. Binding of imidazole to the three mutants is

biphasic, just as the binding of cyanide. The high-affinity phase is designated by the equilibrium dissociation constant, K_{D1} , and the low-affinity phase designated by K_{D2} . The triple mutants have significantly greater affinity for imidazole with both K_{D1} and K_{D2} substantially smaller than K_D for the CcP/imidazole complex. At pH 7, the values of K_{D1} for the triple mutants range from 83 μ M for CcP(triVal) to 0.60 mM for CcP(triLeu), showing increased imidazole binding affinity of 3.8 to 4.7 orders of magnitude. Even the low-affinity binding phases of the triple mutants bind imidazole more strongly than wild-type CcP with K_{D2} values ranging from 12 to 17 mM at pH 7, some 2.5 orders of magnitude smaller than K_D for the CcP/imidazole complex [8].

The pH dependence of imidazole binding to the CcP triple mutants has also been investigated, with the values of K_{D1} and K_{D2} determined between pH 4 and 8 and the kinetics of binding investigated over the same pH region. Finally, the binding of 1-methylimidazole and 4-nitroimidazole to each of the triple mutants has been determined at pH 7.

2. Materials and Methods

2.1. Cloning, Expression, and Purification of CcP Mutants

The expression system for the recombinant CcP (rCcP) used in this study as well as the protocols for construction, expression and purification of the CcP mutants have been previously described [7,8].

2.2. Other Materials

Potassium acetate and potassium phosphate salts were obtained from Fisher Scientific. Imidazole, 1-methylimidazole and 4-nitroimidazole were obtained from Aldrich Chemical Co. Buffers, pH 4.0 to 5.5, 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 an ionic strength of 0.100 M.

2.3. Spectroscopic Measurements and Protein Concentration Determination

Protein spectra were determined as described previously [9]. Concentration of stock protein solutions were determined spectrophotometrically at pH 6.0 using the following extinction coefficients in the Soret band: yCcP, 98 $\text{mM}^{-1} \text{cm}^{-1}$ at 408 nm; rCcP, 101 $\text{mM}^{-1} \text{cm}^{-1}$ at 408 nm; CcP(triAla), 110 $\text{mM}^{-1} \text{cm}^{-1}$ at 406 nm; CcP(triVal), 76 $\text{mM}^{-1} \text{cm}^{-1}$ at 406 nm; and CcP(triLeu), 93 $\text{mM}^{-1} \text{cm}^{-1}$ at 400 nm [7,8].

2.4. Equilibrium Binding and Kinetic Measurements

Equilibrium binding studies were performed with the CcP triple mutants as described in the preceding paper for wild-type CcP and CcP(H52L) [8]. Kinetic studies were carried out using an Applied Photophysics Model DX.17V stopped-flow spectrophotometer. Protein concentrations were typically 1 to 2 μ M and the ligand concentrations varied with the reaction but were always 10-fold or greater than the protein concentration. Data were acquired as a minimum of five different ligand concentrations that varied by at least a factor of 5. The data were fit to either a single or double exponential equation using the

instrument's software as appropriate. Reported rate constants are the average values from at least 10 absorbance vs time scans.

3. Results

3.1. Equilibrium Binding of Imidazole to CcP(triAla)

Imidazole binding to CcP(triAla) is much stronger than binding to wild-type CcP, with essentially complete formation of the imidazole complex at 0.10 M imidazole. Fig. 1 shows the titration of CcP(triAla) at pH 7.0. The Soret maximum shifts from 410 to 413 nm with increasing imidazole concentrations and is accompanied by a substantial increase in the absorbance at 413 nm. Fig. 2 shows plots of the absorbance change at 414 nm as a function of imidazole concentration for the titration of CcP(triAla) at pH 5.5 and 7.0. Both plots are biphasic, with the biphasic character of the titration more noticeable at pH 5.5. Observation of a biphasic equilibrium titration curve suggests two conformations of CcP(triAla) with different imidazole affinities and which do not interconvert on the time scale of the equilibrium experiments. The change in absorbance at 414 nm was fit to Eq. 1, using non-linear least-

$$\Delta A = \frac{\Delta A_1 [L]}{K_{D1} + [L]} + \frac{\Delta A_2 [L]}{K_{D2} + [L]} \quad (1)$$

squares regression, where K_{D1} and K_{D2} represent the equilibrium dissociation constants for the high- and low-affinity phases, respectively. At pH 7.0, the best-fit values for K_{D1} and K_{D2} for the CcP(triAla)/imidazole complexes are 0.22 ± 0.05 mM and 12 ± 1 mM, respectively. Values of K_{D1} and K_{D2} at pH 7.0 are collected in Table 1.

Values for K_{D1} and K_{D2} were determined at each half pH unit between pH 4.0 and 8.0, Fig. 3. K_{D1} and K_{D2} values as a function of pH are tabulated in Table S1 of Appendix A, Supplementary Material provided with this article. Between pH 4 and 8, the low-affinity phase is the major phase of imidazole binding, accounting for 75 ± 8 % of the absorbance change at 414.

Both K_{D1} and K_{D2} are pH dependent with the binding strongest at alkaline pH. The pH dependence of K_{D1} and K_{D2} can be accounted for by the ionization of a single group, Eq. 2.

$$K_{Di} = \frac{(1 + \frac{[H^+]}{K_{ai}})}{(\frac{1}{K_{Di}^{acid}} + \frac{[H^+]}{K_{Di}^{base} K_{ai}})} \quad (2)$$

In Eq. 2, K_{Di}^{acid} and K_{Di}^{base} are the low and high pH limits of K_{Di} , where i is either 1 or 2, and K_{ai} is the acid dissociation constant for the ionizable group affecting K_{Di} . Fitting K_{D2} to Eq. 2 gives best-fit values of 570 ± 80 mM and 6.4 ± 0.7 mM, for K_{D2}^{acid} and K_{D2}^{base} , respectively, Table 2. The ionizable group has a pK_{a2} of 6.8 ± 0.1 . The values of K_{D1} show more scatter than those of K_{D2} due to the smaller amplitude in the titration plots. Best-fit values of K_{D1}^{acid} , K_{D1}^{base} and the pK_{a1} value for the group influencing K_{D1} are collected in Table 2.

The spectrum for 100% formation of the CcP(triAla)/imidazole complex can be calculated from the data shown in Figs. 1 and 2. The spectrum of the CcP(triAla)/imidazole complex is shown in Fig. S1 of the supplementary data and selected spectral parameters are collected in Table 3. The CcP(triAla)/imidazole complex has a Soret maximum at 413 nm with an extinction coefficient of $143 \text{ mM}^{-1} \text{ cm}^{-1}$ and α (shoulder) and β bands at 564 and 536 nm, respectively.

3.2. Kinetics of Imidazole Binding to CcP(triAla)

Binding of imidazole to CcP(triAla) was followed by stopped-flow spectrophotometry at 414 nm using pseudo-first-order conditions with imidazole in excess. Binding of imidazole to CcP(triAla) is biphasic, with the observed rate constants for the fast and slow phases of the reaction designated k_{fast} and k_{slow} , respectively. The rate constant for the fast phase of the reaction is linearly dependent upon the imidazole concentration, Fig. S2 in the supplementary data, while k_{slow} is independent of ligand concentration.

Observed rate constants that are linearly dependent upon ligand concentration are generally attributed to the binding step where the observed rate constant is a function of both the apparent association, K_a^{app} , and dissociation, K_d^{app} , rate constants for the enzyme ligand complex, Eq. 3.

$$k_{fast} = k_a^{app} [L] + k_d^{app} \quad (3)$$

The apparent association and dissociation rate constants can be determined from the slope and intercept of plots such as that shown in Fig. S2. Observed rate constants that are independent of ligand concentration such as k_{slow} are typically associated with conformational changes within the protein or protein-ligand complex that limit the rate. We define the rate-limiting unimolecular rate constant k_{max} . For the slow phases of the CcP(triAla) and CcP(triLeu) imidazole reactions, we equate k_{slow} with k_{max} . Values of k_a^{app} , k_d^{app} , and k_{max} for the fast and slow phases of imidazole binding to CcP(triAla) at pH 7.0 are collected in Table 4.

The rate constants k_a^{app} , k_d^{app} , and k_{max} have been determined for the CcP(triAla)/imidazole reaction as a function of pH and are shown in Fig. 4. The apparent association rate constant increases with increasing pH while k_d^{app} and k_{max} are essentially independent upon pH. Values of k_a^{app} , k_d^{app} , and k_{max} are tabulated in Table S2 of the supplemental data. The average values for k_d^{app} and k_{max} over the pH range 4.0 to 8.0 are $0.47 \pm 0.10 \text{ s}^{-1}$ and $(3.2 \pm 1.1) \times 10^{-2} \text{ s}^{-1}$, respectively. The pH dependence of k_a^{app} can be attributed to the ionization of a single group but we will see later that k_a^{app} for the fast phase of the CcP(triLeu)/imidazole reaction is influenced by two ionizable groups. We choose to fit the CcP(triAla) data to an equation representing two ionizable groups with the proviso that ionization of the second group does not influence the CcP(triAla) data between pH 4 and 8. An equation describing the influence of two ionizable groups on the apparent rate constant is shown in Eq. 4. In Eq.4, k_a^{acid} , k_a^{neut} , and k_a^{base}

$$k_a^{app} = \frac{k_a^{acid} \frac{[H^+]}{K_{a1}} + k_a^{neut} + k_a^{base} \frac{K_{a2}}{[H^+]}}{\frac{[H^+]}{K_{a1}} + 1 + \frac{K_{a2}}{[H^+]}} \quad (4)$$

are the low-, intermediate, and high-pH values of k_a^{app} , while K_{a1} and K_{a2} are the acid dissociation constants for the ionizable groups that influence the reaction. For the CcP(triAla) data, either k_a^{neut} equals k_a^{base} or pK_{a2} is greater than 9 such that it does not influence the data at pH 8. Non-linear least squares regression was used to determine the best-fit values for k_a^{acid} , k_a^{neut} , and the pK_{a1} value for the more acidic ionizable group. The best-fit parameters are collected in Table 5.

The ratio of k_d^{app}/k_a^{app} defines a kinetically determined equilibrium dissociation constant, K_D^{kin} . Over the pH range 4.0 to 8.0, the calculated value of K_D^{kin} is essentially identical to the experimentally determined low-affinity equilibrium dissociation constant, K_{D2} , for the CcP(triAla)/imidazole complex. Fig. S3 of the supplementary data shows a comparison of K_D^{kin} and K_{D2} . The near identity of K_D^{kin} and K_{D2} identifies the fast kinetic phase of the CcP(triAla)/imidazole reaction with binding of imidazole to the low-affinity conformation of CcP(triAla). Therefore, the slow kinetic phase of the reaction is attributed to the binding of imidazole to the high-affinity conformation of CcP(triAla) and this binding is limited by an isomerization within the high-affinity conformation characterized by $k_{slow} = k_{max}$.

3.3. Equilibrium Binding of Imidazole to CcP(triLeu)

The binding of imidazole to CcP(triLeu) is, in general, similar to the binding of imidazole to CcP(triAla). Fig. 5 shows a spectroscopic titration of CcP(triLeu) with imidazole at pH 7.0. The Soret maximum shifts from 405 to 412 nm during the titration with a substantial increase in absorptivity. A plot of the change in absorbance as a function of imidazole concentration, Fig. S4 supplementary data, is biphasic. Fitting the absorbance changes to Eq. 1 gives best-fit values of 0.60 ± 0.04 mM and 17 ± 7 mM for K_{D1} and K_{D2} , respectively, Table 1. The values for K_{D1} and K_{D2} are slightly larger than those for CcP(triAla) indicating somewhat weaker binding of imidazole by CcP(triLeu). However, the high-affinity imidazole binding phase of CcP(triLeu) is the dominant phase, accounting for 78% of absorbance change at pH 7.

In contrast to imidazole binding to CcP(triAla), binding of imidazole to CcP(triLeu) is monophasic between pH 4.0 and 6.5 and biphasic between pH 7.0 and 8.0, Fig. 6. At lower pH, the experimental equilibrium constant is consistent with binding to the high-affinity conformation of CcP(triLeu). Binding to the low-affinity conformation of CcP(triLeu) can only be detected between pH 7 and 8, where it accounts for an average of 30 ± 7 % of the absorbance change at 414 nm. Values of K_{D1} and K_{D2} are tabulated in Table S3 of the supplementary data.

The pH dependence of K_{D1} can be attributed to the effects of a single ionizable group just as for the CcP(triAla)/imidazole reaction. Fitting K_{D1} to Eq. 2 gives best-fit values of 120 ± 60 mM and 0.22 ± 0.15 mM, for K_{D1}^{acid} and K_{D1}^{base} , respectively, Table 2. The ionizable group has a pK_a of 7.5 ± 0.4 .

The spectrum for 100% formation of the CcP(triLeu)/imidazole complex can be calculated from the data shown in Figs. 5 and S4. The spectrum of the CcP(triLeu)/imidazole complex is shown in Fig. S1 of the supplementary data and selected spectral parameters are collected in Table 3. The CcP(triLeu)/imidazole complex has a Soret maximum at 412 nm with an extinction coefficient of $124 \text{ mM}^{-1} \text{ cm}^{-1}$ and α (shoulder) and β bands at 564 and 532 nm, respectively.

3.4. Kinetics of Imidazole Binding to CcP(triLeu)

The kinetics of imidazole binding to CcP(triLeu) is biphasic over the pH range 4.0 to 8.0 even though the equilibrium titrations are monophasic between pH 4.0 and 6.5 (Fig. 6). The rate constant for the fast phase of the reaction is linearly dependent upon the imidazole concentration, Fig. S5 supplementary data, from which values of k_a^{app} and k_d^{app} can be extracted and $k_{slow} = k_{max}$ is independent of ligand concentration. Values of k_a^{app} , k_d^{app} , and k_{max} for the CcP(triLeu) imidazole reaction were determined at each half pH between pH 4.0 and 8.0, Table S4 supplementary data, and plotted in Fig. 7.

Although the pH dependencies of the CcP(triLeu) and CcP(triAla) reactions are similar (Fig. 4 and Fig. 7) there are some differences. While k_a^{app} for the CcP(triAla)/imidazole reaction appears to be influenced by a single ionizable group, k_a^{app} for the CcP(triLeu)/imidazole reaction has a maximum value near pH 7 indicating that at least two ionizable groups affect the reaction. The pH dependence of k_a^{app} for the CcP(triLeu)/imidazole reaction can be described by Eq. 4 where both ionizable groups influence the data. The best-fit values for the parameters defined by Eq. 4 are collected in Table 5. In order to get a reasonable fit we had to set $pK_{a1} = pK_{a2}$ and under these conditions, the values of pK_{a1} and pK_{a2} converge to the same value of 7.3. The system is not well defined, primarily because we cannot determine the high pH limit of the association rate constant, and the estimated error in the parameters is large. Interestingly, and providing some support for the accuracy of the fitting procedure, is that the values for K_a^{acid} and K_a^{neut} are identical to those for the CcP(triAla) reaction, Table 5.

The value of k_d^{app} is independent of pH with an average value of $0.38 \pm 0.12 \text{ s}^{-1}$. However, $k_{slow} = k_{max}$ has a small pH dependence and we fit the pH dependence to an equation analogous to that of Eq. 4 with the proviso that the second ionization does not influence the reaction. Best-fit values for k^{acid} and k^{neut} and the pK_{a1} value that influences k_{max} are collected in Table 5. If we just average k_{max} over the pH range 4 to 8 the average value is $0.048 \pm 0.018 \text{ s}^{-1}$, similar to the value for the CcP(triAla) reaction.

Over the pH range 7.0 to 8.0, the calculated values of K_D^{kin} are similar to those of K_{D2} determined from the equilibrium titrations and about a factor of 10 weaker than the K_{D1} values in this same pH region, Fig 6. Equating K_D^{kin} with K_{D2} provides values of K_{D2} between pH 4 and 6.5, where equilibrium values of K_{D2} could not be extracted from the titration curves. Fitting a composite data set consisting of the K_{D2} equilibrium data and the K_D^{kin} data to Eq. 2 gives best-fit values of $160 \pm 60 \text{ mM}$ and $9.4 \pm 3.1 \text{ mM}$, for K_{D2}^{acid} and K_{D2}^{base} , respectively. The ionizable group has a pK_a of 6.8 ± 0.4 , Table 2.

3.5. Equilibrium Binding of Imidazole to CcP(triVal)

Equilibrium binding of imidazole to CcP(triVal) is similar to the binding of imidazole to CcP(triAla). Fig. 8 shows a spectroscopic titration of CcP(triVal) with imidazole at pH 7.0. The Soret maximum shifts from 406 to 412 nm during the titration with a substantial increase in absorptivity. A plot of the change in absorbance as a function of imidazole concentration, Fig. S6 supplementary data, is biphasic. Fitting the absorbance changes to Eq. 1 gives best-fit values of $83 \pm 5 \mu\text{M}$ and $12 \pm 4 \text{ mM}$ for K_{D1} and K_{D2} , respectively, Table 1. The high affinity phase of the binding accounts for 68% of the absorbance change in the Soret region at pH 7. In addition, the high-affinity phase for imidazole binding to CcP(triVal) is the strongest imidazole binding for any CcP mutant we have investigated, 4.7 orders of magnitude stronger than for wild-type CcP and less than 50-times weaker than the strongest reported binding of imidazole binding to any heme protein [15].

The pH dependence of K_{D1} and K_{D2} for the CcP(triVal)/imidazole reaction is shown in Fig. 9 and values are tabulated in Table S5 of the supplementary data. The pH dependence of both K_{D1} and K_{D2} can be attributed to the effects of a single ionizable group and fit to Eq. 2. Best-fit values for the parameters defined in Eq. 2 are collected in Table 2. The high-affinity phase of binding gives best-fit values of $9.6 \pm 5.5 \text{ mM}$ and $96 \pm 7 \mu\text{M}$, for K_{D1}^{acid} and K_{D1}^{base} , respectively, and a pK_a of 5.7 ± 0.1 for the ionizable group, Table 2. The low-affinity phase of binding gives best fit values of $0.38 \pm 0.39 \text{ M}$ and $6.7 \pm 2.3 \text{ mM}$, for K_{D1}^{acid} and K_{D1}^{base} , respectively, and a pK_a of 6.2 ± 0.3 for the ionizable group, Table 2. The high affinity phase accounts for an average of $67 \pm 10\%$ of the absorbance change over the pH range 4 to 8.

The spectrum for 100% formation of the CcP(triVal)/imidazole complex can be calculated from the data shown in Figs. 8 and S6. The spectrum of the CcP(triVal)/imidazole complex is shown in Fig. S1 of the supplementary data and selected spectral parameters are collected in Table 3.

3.6. Kinetics of Imidazole Binding to CcP(triVal)

The binding of imidazole to CcP(triVal) is biphasic but in contrast with the CcP(triAla) and CcP(triLeu) reactions, both k_{fast} and k_{slow} are dependent upon the imidazole concentration, increasing linearly at low concentrations but reaching a limiting value at high ligand concentrations, Fig. S7, supplementary data. In this case three parameters are required to fit the imidazole concentration dependence of both k_{fast} and k_{slow} . To compare the CcP(triVal) imidazole kinetic parameters with those for CcP(triAla) and CcP(triLeu), we use k_a^{app} , k_d^{app} , and a maximum rate constant, k_{max} , which is the limiting rate at infinite imidazole concentration, to define an empirical equation shown in Eq. 5. The actual interpretation of the parameters will

$$k_{obs} = \frac{k_a^{app}[L] + k_d^{app}}{\frac{k_a^{app}}{k_{max}}[L] + 1} \quad (5)$$

depend upon the mechanism of the reaction. The pH dependence of k_a^{app} , k_d^{app} , and k_{max} for both reaction phases of imidazole binding to CcP(triVal) are shown in Fig. 10 and values are tabulated in Tables S6 and S7, Supplementary Material.

A comparison of Figs. 4, 7, and 10 shows that the pH dependence and absolute values of k_a^{app} for the CcP(triAla) and CcP(triLeu) reactions are similar to the values of k_a^{app} for both phases of the CcP(triVal)/imidazole reaction. Likewise, k_d^{app} for the CcP(triAla) and CcP(triLeu) reactions and k_d^{app} for the fast-phase of the CcP(triVal) reactions are all independent of pH with similar magnitudes: 0.47 ± 0.10 , 0.38 ± 0.12 , and $0.25 \pm 0.08 \text{ s}^{-1}$, respectively. k_d^{app} for the slow phase of the CcP(triVal) reactions has a small pH dependence, which we fit to Eq.4 and include the best-fit parameters in Table 5. The low-pH limit of k_d^{app} for the slow phase of the CcP(triVal) reaction is $0.026 \pm 0.009 \text{ s}^{-1}$ and the high-pH limit is $0.073 \pm 0.011 \text{ s}^{-1}$. The apparent pK_a for the transition between low and high pH rates is 6.1 ± 0.5 .

The maximum rates observed for both phases of the CcP(triVal)/imidazole reaction, k_{max} , are similar to $k_{slow} = k_{max}$ for the CcP(triAla) reaction in that all three are pH and concentration independent. The magnitudes vary by about a factor of 50 and this may be the reason for the different concentration dependencies for the rates of imidazole binding to the triple mutants. The values of k_{max} for the fast and slow phases of the CcP(triVal) reaction and slow phase of the CcP(triAla) reactions are 1.5 ± 0.3 , 0.17 ± 0.04 , and $0.032 \pm 0.011 \text{ s}^{-1}$, respectively. $k_{slow} = k_{max}$ for the CcP(triLeu)/imidazole reaction in concentration independent but does have a small pH dependence; it varies from $0.032 \pm 0.003 \text{ s}^{-1}$ at low pH to $0.077 \pm 0.011 \text{ s}^{-1}$ at high pH. The apparent pK_a for the transition between low- and high-pH rates is 6.7 ± 0.4 .

3.7. Binding of 1-Methylimidazole Binding to CcP(triAla), CcP(triLeu), and CcP(triVal) at pH 7

Just as with imidazole, 1-methylimidazole (MIM) binds to CcP(triAla), CcP(triVal), and CcP(triLeu) more strongly than it binds to yCcP. Fig. 11 shows the CcP(triLeu)/MIM titration while Figs S8 and S9 show the CcP(triAla)/MIM and CcP(triVal)/MIM titrations at pH 7.0. The binding of MIM to CcP(triLeu) is monophasic, Fig. S10, with a K_D value of $39 \pm 3 \text{ mM}$. This is about twice as large as K_{D2} for imidazole binding to CcP(triLeu), Table 1, and we associate MIM binding to the low affinity conformation of CcP(triLeu). The titrations of CcP(triAla) and CcP(triVal) are biphasic, Figs. S11 and S12. For CcP(triAla), the low affinity phase, with a K_{D2} of $0.11 \pm 0.01 \text{ M}$, is the dominant phase, accounting for about 70% of the absorbance change in the Soret region. K_{D1} for the CcP(triAla)/MIM reaction is $0.35 \pm 0.34 \text{ mM}$. The high-affinity phase is the dominant phase in the CcP(triVal)/MIM titration accounting for 77% of the absorbance change in the Soret region. K_{D1} and K_{D2} values are $0.16 \pm 0.04 \text{ mM}$ and $6.8 \pm 3.3 \text{ mM}$, respectively, Table 1.

The spectra for 100% complex formation for the MIM complexes of CcP(triAla), CcP(triVal), and CcP(triLeu) can be calculated from the titration data and selected spectral parameters are collected in Table 3.

3.8. Binding of 4-Nitroimidazole to CcP(triAla), CcP(triLeu), and CcP(triVal) at pH 7.0

The spectroscopic titration of CcP(triVal) by 4-nitroimidazole is shown in Fig. 12 while the CcP(triAla)/4NI and CcP(triLeu)/4NI titrations are shown in Figs. S13 and S14 of the supplemental data. Addition of saturated solution of 4NI to CcP(triVal) causes a 5 nm red-

shift of the Soret band with a 34% increase in absorbance, consistent with formation of a six-coordinate low-spin heme complex. Over the limited 4NI concentration range available (up to about 5 mM), 4NI binds to CcP(triVal) in a monophasic manner, Fig. S15, with a K_D value of 0.56 ± 0.02 mM, Table 1. The spectrum for complete formation of the CcP(triVal)/4NI complex can be calculated from the data in Figs. 12 and S15. Selected spectroscopic parameters are collected in Table 3.

Spectroscopic changes associated with adding saturated 4NI to CcP(triAla), Fig. S13, and CcP(triLeu), Fig. S14, are much smaller than the changes observed by adding 4NI to CcP(triVal). This may be because high-affinity binding of 4NI is not evident for CcP(triAla) and CcP(triLeu) while binding of 4NI to CcP(triVal) is primarily to the high-affinity conformation. Assuming that the spectrum of the 4NI complex is similar for the high- and low-affinity conformations of the CcP triple mutants and using the spectra of the CcP(triVal)/4NI and metMb/4NI complexes as references, we can estimate K_{D2} values for binding of 4NI to CcP(triAla) and CcP(triLeu) of 16 ± 11 and 35 ± 13 mM, respectively, Table 1.

4. Discussion

4.1. Spectroscopic Properties of the Imidazole-CcP Complexes at pH 7

The electronic absorption spectra of twelve imidazole/CcP complexes were determined at pH 7, Table 3. These include the imidazole derivatives of yCcP, rCcP, CcP(triAla), CcP(triVal), CcP(triLeu), the 1-methylimidazole derivatives of yCcP, rCcP, CcP(H52L), CcP(triAla), CcP(triVal), CcP(triLeu), and the 4-nitroimidazole derivative of CcP(triVal). We were unable to obtain the spectra of the imidazole/CcP(H52L) complex and the 4-nitroimidazole complexes of yCcP, CcP(H52L), CcP(triAla) and CcP(triLeu) due to the weak affinity for these protein ligand combinations. In some cases the UV regions of the spectra were not determined due to the high absorbance of the ligands at the concentrations necessary to saturate the proteins.

A major consideration in interpreting the spectrum of imidazole complexes is to determine if the bound imidazole is present in its neutral form or as the imidazolate anion. Binding of imidazole to the heme iron can significantly increase the acidity of the bound ligand promoting imidazolate formation. Model studies have shown that the electronic absorption spectrum of imidazole/heme complexes can distinguish between imidazole and imidazolate binding. The Soret bands of imidazolate/heme complexes are red-shifted with a less intense Soret band compared to the Soret bands of imidazole/heme complexes [8,15–18].

4.1.1. Spectroscopic Properties of the 1-Methylimidazole Complexes of CcP and the CcP Mutants at pH 7—1-Methylimidazole binding to yCcP and the four distal pocket CcP mutants is sufficiently strong that spectra for 100% complex formation can be determined for all six proteins at pH 7, Table 3. MIM cannot ionize to form imidazolate so the observed spectra are due to binding of neutral imidazole to the heme. The Soret bands for the seven MIM complexes listed in Table 3 vary between 414 and 420 nm. The Soret bands of all three CcP triple mutant/MIM complexes are at 414 nm, the band for the yCcP/MIM complex occurs at 416 nm, similar to the band position at 417 nm for the

metMb/MIM complex. The MIM complexes of rCcP and CcP(H52L) have Soret bands at 420 nm. The extinction coefficients of the CcP and CcP mutant MIM complexes range between 117 and 138 $\text{mM}^{-1} \text{cm}^{-1}$ at the Soret maxima, approaching the value of 141 $\text{mM}^{-1} \text{cm}^{-1}$ for the metMb/MIM complex, Table 3.

In addition to the changes in the Soret band, the visible region of the spectra show the characteristic changes associated with formation of six-coordinate, low-spin complexes of the heme iron. The heme group in yCcP is predominantly five-coordinate, high-spin with prominent charge-transfer bands near 508 and 645 nm. Upon binding of MIM, the charge-transfer bands diminish in intensity and are replaced by prominent α and β bands, with the β band dominating the visible region of the spectrum. The β band positions for the yCcP/MIM and the CcP(H52L)/MIM complexes are at 542 and 544 nm, respectively, while the β band positions for the three triple mutant/MIM complexes are between 534 and 536 nm, similar to the β band of the metMb/MIM complex.

4.1.2. Spectroscopic Properties of the Imidazole Complexes of CcP and the CcP Mutants at pH 7—

Binding of imidazole to the heme iron significantly increases the acidity of the ligand and we need to consider the possibility that bound imidazole can ionize to the imidazolate anion. Binding of imidazole to yCcP is unusual in that there is a decrease in the maximum extinction coefficient from 98 to 85 $\text{mM}^{-1} \text{cm}^{-1}$, accompanied by a shift in the Soret maximum from 408 to 412 nm [8]. More importantly, the largest increase in the difference spectrum between the imidazole complex and wild-type yCcP occurs at 436 nm. As discussed in the preceding paper [8], the increase in absorbance near 436 nm is a signature for imidazolate binding and we concluded that the ligand in the yCcP/imidazole complex is between 22 and 32% ionized at pH 7.

The binding of imidazole to wild-type CcP and the four CcP mutants cause a 4 to 7 nm red-shift in the position of the Soret band but the absorptivity changes of the imidazole complexes fall into two groups. The spectra of the yCcP and CcP(H52L) imidazole complexes show the largest increases in absorptivity at 436 and 442 nm, respectively [8], while the imidazole complexes of rCcP [8] and the triple mutants, Figs. 1, 5, and 8, show the largest increases in absorptivity between 415 and 420 nm. The spectra of the imidazole complexes of the CcP triple mutant look very much like the CcP/MIM complexes, Figs. 11, S8, and S9, and we conclude that the neutral form of imidazole is bound to the triple mutants at pH 7. The apolar nature of the distal heme pocket in the CcP triple mutants inhibits ionization of the bound imidazole and the net positive charge on the Fe(III) heme is largely compensated by the negatively-charged Asp-235 in the proximal heme pocket.

4.1.3. Spectroscopic Properties of the 4-Nitroimidazole Complexes of the CcP Triple Mutants at pH 7—

Binding of 4-nitroimidazole to yCcP, CcP(H52L), CcP(triAla), and CcP(triLeu) is too weak to determine the spectrum of the 4NI complexes for these proteins. The CcP(triVal) binds 4NI three orders of magnitude more strongly than yCcP and the spectrum of the CcP(triVal)/4NI complexes could be determined. The Soret band for the CcP(triVal)/4NI complex occurs at 416 nm with an extinction coefficient of 110 $\text{mM}^{-1} \text{cm}^{-1}$, Table 3, similar to the spectroscopic parameters for the metMb/4NI complex, Table 3 [19]. There is no evidence for bound imidazolate in any of the three triple mutants, with the

largest increase in the difference spectra occurring between 418 and 420 nm, Figs. 12, S13, and S14. In spite of the very acidic nature of 4NI, with a pK_a of 9.5 for the imidazole/imidazolate ionization in the free ligand, the apolar nature of the distal heme pocket in the CcP triple mutants destabilizes formation of a negatively-charged bound ligand compared to the neutral ligand.

4.2 Mechanism of Imidazole Binding to the CcP Triple Mutants

The major finding of this work is that making the distal heme pocket in CcP more apolar significantly enhances CcP's affinity for imidazole, MIM and 4NI. Although the binding of imidazole to all three triple mutants is biphasic, even the low-affinity binding phase has imidazole affinities that are 240- to 330-fold greater than wild-type CcP, Table 1. The high-affinity forms of the triple mutants bind imidazole with 6,700- to 48,000-fold greater affinity than CcP, an almost 5-order of magnitude increase in imidazole affinity for CcP(triVal).

The properties of the CcP triple mutants are complex [7,8] and a more detailed consideration of imidazole binding can provide further characterization of these mutants. In addition to the biphasic equilibrium binding curves, the binding kinetics are biphasic as well. CcP(triAla) and CcP(triLeu) have a fast kinetic phase that is linearly dependent upon the imidazole concentration and a slow kinetic phase that is independent of ligand concentration. On the other hand, both phases of imidazole binding to CcP(triVal) are hyperbolic functions of the imidazole concentration, Fig. S7. A previous study of cyanide binding to the three CcP triple mutants show exactly the same equilibrium and kinetic behavior [7], indicating that these properties are not unique to imidazole binding but properties of the mutants.

The biphasic nature of the equilibrium titration curves indicate that each of the CcP triple mutants exist in at least two conformations with different ligand affinity. The conformations do not interconvert on the time scale of the equilibrium experiments and each conformation can be treated as independent species in solution. The saturation kinetics for both phases of ligand binding to CcP(triVal) indicate that an unimolecular step limits the rate of product formation. The two most common mechanisms for this type of kinetic behavior are either formation of a precursor complex, followed by a unimolecular conversion to the final product or the presence of a closed form of the enzyme in which the rate of opening limits the binding rate. At the moment, we cannot distinguish between these two mechanisms. We will use the precursor complex mechanism to discuss the equilibrium and kinetic properties of imidazole binding to the CcP triple mutants in this section. A consideration of the closed conformation mechanism is given in the supplementary data. The precursor complex mechanism is shown in Eq. 6. To be consistent with the experimental observations, formation of the precursor complex cannot be associated

with significant spectroscopic changes, meaning that the ligand is not bound to the heme in EL^* , rather heme binding occurs in the EL^*/EL isomerization step. In order to convert all of the enzyme to the heme-bound imidazole complex, as suggested by the large extinction

coefficients in the Soret region for the final complexes, Table 3, k_3 must be significantly larger than k_4 .

Assuming that the EL* is in a steady-state during the reaction, the observed rate constant is described by Eq. 5 above. The apparent kinetic parameters are expressed in terms of the rate constants defined in the mechanism in Eqs. 7 to 9. For CcP(triVal), all three kinetic parameters

$$k_a^{app} = \frac{(k_3 + k_4)}{(k_2 + k_3)} k_1 \quad (7)$$

$$k_d^{app} = \frac{k_2 k_4}{k_2 + k_3} \quad (8)$$

$$k_{max} = (k_3 + k_4) \quad (9)$$

can be determined for both imidazole binding phases, Table 4. The kinetics of imidazole binding to CcP(triAla) and CcP(triLeu) are special cases of Eq. 5. The fast kinetic phases for CcP(triAla) and CcP(triLeu) are linearly dependent upon the ligand concentration, consistent with Eq. 5 if the first term in the denominator is very small compared to the second. In this case k_a^{app} and k_d^{app} can be determined from the slope and intercept of a plot of k_{obs} versus imidazole concentration for the low-affinity phase of imidazole binding, Table 4. The slow kinetic phases for CcP(triAla) and CcP(triLeu) are independent of ligand concentration, consistent with Eq. 5 if the first terms in both the numerator and denominator are very large compared to the second terms. In this case, k_{slow} equals k_{max} for the high-affinity imidazole binding phase of the reaction, Table 4.

At pH 7, binding of imidazole to the low-affinity conformation of the CcP triple mutants is similar to that of imidazole binding to metmyoglobin [10–13]. The K_{D2} values for the triple mutants are about a factor of two smaller than K_D for metmyoglobin and the apparent rate constants for the CcP mutants are also somewhat smaller than those for metmyoglobin. The apparent association rate constant, k_a^{app} , for the CcP triple mutants varies between 36 and 170 M⁻¹s⁻¹ while the reported values of k_a for metmyoglobin range between 170 and 310 M⁻¹s⁻¹ at pH 7 [10–13]. For the precursor complex mechanism to be consistent with observation, k_3 must be much larger than both k_2 and k_4 and under these circumstances Eq. 7 predicts that k_a^{app} will be equal to k_1 , the true association rate constant for formation of the precursor complex. Under conditions where k_{fast} is linearly dependent on imidazole concentration, this will also be the rate of formation of the final complex so the comparison of k_a^{app} for the triple mutants with k_a for the metmyoglobin reactions is reasonable.

The apparent dissociation rate constant, k_d^{app} , for the triple mutants varies between 0.24 and 0.43 s⁻¹ while k_d for metmyoglobin ranges between 4.5 and 8.7 s⁻¹. The lower values of k_d^{app} for the triple mutants compared to metmyoglobin can be at least partly attributable to the observation that k_d^{app} provides a lower limit for the true ligand dissociation rate from the precursor complex, k_2 , since the $k_4/(k_2 + k_3)$ term must be much less than 1.

The pH dependencies of both the apparent association and dissociation rate constants for metmyoglobin and the low-affinity conformations of the CcP triple mutants are similar, with the association rate constant increasing with increasing pH and the dissociation rate constant independent of pH. The major difference between imidazole binding to the low-affinity conformations of the CcP triple mutants and to metmyoglobin is that conformational transitions limit the maximum rate of imidazole binding to the CcP triple mutants and also the rate of ligand dissociation.

4.3 Conclusions

The CcP triple mutants, with their apolar distal heme pockets, have very interesting properties. Their spectroscopic properties are pH dependent, with the low pH forms having predominantly five-coordinate, high-spin hemes and the high-pH forms having predominantly six-coordinate, low-spin hemes [8]. Ligand binding studies indicate that all three triple mutants have at least two independent conformational forms that have differential ligand affinity. Binding of imidazole, 1-methylimidazole, and 4-nitroimidazole to the low-affinity conformations is about two- to three-orders of magnitude stronger than binding to wild-type CcP and is similar to the binding of these ligands to metmyoglobin. The high-affinity conformations bind imidazole up to 4.7 orders of magnitude stronger than wild-type CcP. While imidazole binding is enhanced in the triple mutants, cyanide binding is severely inhibited [7]. The high- and low-affinity conformations of the CcP triple mutants bind cyanide three to five orders of magnitude weaker than wild-type CcP at pH 7 [7,20]. These dramatic differences in ligand binding properties of the CcP triple mutants compared to wild-type CcP are also reflected in the catalytic activity of the enzyme. The reaction between CcP and hydrogen peroxide mimics the binding of HCN, both requiring base catalysis from the distal histidine to bind to the heme iron. Due to a lack of the distal histidine residue, the CcP triple mutants react very slowly with HCN and with hydrogen peroxide, having bimolecular rate constants that are three to seven orders of magnitude smaller than that of wild-type CcP. The low rate of reaction with hydrogen peroxide leads to substantially decreased peroxidase activity of the triple mutants, less than 0.02% under normal assay conditions [7]. On the other hand, due to increased binding of small, apolar organic substrates within the distal heme pocket of the triple mutants, the non-native peroxygenase activity is increased up to 34-fold [9,21].

Supplementary Material

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Abbreviations

CcP	generic abbreviation for cytochrome <i>c</i> peroxidase
yCcP	authentic yeast cytochrome <i>c</i> peroxidase isolated from <i>S. cerevisiae</i>
rCcP	recombinant cytochrome <i>c</i> peroxidase expressed in <i>E. coli</i> with an amino acid sequence identical to that of yCcP
CcP(TriAla)	a triple point mutation of rCcP with R48A/W51A/H52A

CcP(TriVal)	a triple point mutation with R48V/W51V/H52V
CcP(TriLeu)	a triple point mutation of rCcP with R48L/W51L/H52L
MIM	1-methylimidazole
4NI	4-nitroimidazole

Appendix A. Supplementary data

Supplementary material associated with this article can be found in the online version at doi:

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Highlights

- Imidazole binding to the apolar distal pocket mutants of CcP is biphasic
- CcP(triAla) has a 4.7-order of magnitude higher affinity for imidazole than CcP
- Binding of imidazole is pH dependent with apparent pK_A s between 5.6 and 9.4
- Imidazole binding increases between 26- and 470-fold between pH 4 and 8

Figure 1.

Upper Panel: Titration of CcP(triAla) with imidazole at pH 7.0. The Soret band shifts from 410 to 413 nm with a 35% increase in absorptivity as the imidazole concentration increases from 0 to 100 mM. Lower Panel: Difference spectrum between CcP(triAla) in the presence and absence of 100 mM imidazole. Maximum and minimum peaks in the difference spectrum occur at 416 and 376 nm, respectively. The experiment was carried out in a double beam spectrophotometer with compensating imidazole in the reference cuvette to correct for

the imidazole absorbance. [CcP(triAla)] = 10.8 μ M, [imidazole] = 0 to 100 mM, 0.100 M ionic strength potassium phosphate buffer, pH 7.0, 25 °C.

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Figure 2.

Plot of the absorbance change at 414 nm for CcP(triAla) with increasing concentrations of imidazole. Solid circles – pH 7.0; Open circles – pH 5.5. Both curves are biphasic and were analyzed according to Eq. 1 of the text. Best-fit values for K_{D1} are 0.22 ± 0.5 and 0.10 ± 0.03 at pH 7 and 5.5, respectively, and for K_{D2} are 11 ± 1 and 107 ± 16 at pH 7.0 and 5.5, respectively. The low-affinity binding phase accounts for 84% of the absorbance change at pH 7.0 and 76% at pH 5.5. The solid lines were calculated from Eq. 1 using the best-fit parameters. [CcP(triAla)] = 10.8 μ M, [imidazole] = 0 to 200 mM, 0.100 M ionic strength potassium phosphate buffer, 25 $^{\circ}$ C.

Figure 3. pH dependence of the negative logarithm of K_{D1} (solid circles) and K_{D2} (solid circles) for the binding of imidazole to CcP(triAla). The data were fit to Eq. 2 of the text using non-linear least squares regression. K_{D1} increases from 0.13 mM at high pH to > 25 mM at low pH with an apparent pK_a of 5.6 ± 0.5 . K_{D2} increases from 6.4 mM at high pH to 570 mM at low pH with an apparent pK_a of 6.8 ± 0.1 . Best-fit parameters are collected in Table 2. The values of K_D^{kin} (open triangles) are also plotted on the graph to compare with the K_{D2} values.

Figure 4.

pH dependence of the rate constants, k_a^{app} (solid circles), k_d^{app} (open circles), determined from the slope and intercept of a plot of k_{fast} as a function of the imidazole concentration, and k_{max} (solid triangles), which equals the observed rate constant for the slow phase of the reaction for the binding of imidazole to CcP(triAla). k_d^{app} and k_{max} are essentially independent of pH, with average values of $0.47 \pm 0.10 \text{ s}^{-1}$ and $0.032 \pm 0.011 \text{ s}^{-1}$, respectively. The association rate constant, k_a^{app} , increases as a function of pH and was fit to Eq. 4 of the text where only the group with pK_{aI} affects the rate constant. k_a^{app} increases from a value of $2.2 \pm 0.4 \text{ M}^{-1} \text{ s}^{-1}$ at low pH to $99 \pm 25 \text{ M}^{-1} \text{ s}^{-1}$ at high pH with an apparent pK_{aI} of 7.0 ± 0.2 . Best-fit parameters are collected in Table 5. Experimental conditions: 0.10 M ionic strength buffers, 25 °C.

Figure 5.

Upper Panel: Titration of CcP(triLeu) with imidazole at pH 7.0. The Soret band shifts from 405 to 412 nm with a 38% increase in absorptivity as the imidazole concentration increases from 0 to 100 mM. Lower Panel: Difference spectrum between CcP(triLeu) in the presence and absence of 100 mM imidazole. Maximum and minimum peaks in the difference spectrum occur at 415 and 378 nm, respectively. The experiment was carried out in a double beam spectrophotometer with compensating imidazole in the reference cuvette to correct for

the imidazole absorbance. [CcP(triAla)] = 11.1 μ M, [imidazole] = 0 to 100 mM, 0.100 M ionic strength potassium phosphate buffer, pH 7.0, 25 °C.

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Figure 6. pH dependence of the negative logarithm of K_{D1} (solid circles) and K_{D2} (open circles) for the binding of imidazole to CcP(triLeu). K_{D2} could only be determined between pH 7 and 8 in the equilibrium titration curves. Included on the plot are values of K_D^{kin} (open triangles) calculated from the fast kinetic phase of imidazole binding to CcP(triLeu). K_D^{kin} correlates with K_{D2} between pH 7 and 8 and we assume that K_D^{kin} represents K_{D2} throughout the pH range 4 to 8. The data were fit to Eq. 2 of the text using non-linear least squares regression. K_{D1} increases from 0.22 mM at high pH to 120 mM at low pH with an apparent pK_a of 7.5. The combination of K_{D2} and K_D^{kin} increases from 9.4 mM at high pH to 160 mM at low pH with an apparent pK_a of 6.8. Best-fit parameters are collected in Table 2.

Figure 7. pH dependence of the rate constants, k_a^{app} (solid circles), k_d^{app} (open circles), and k_{max} (solid triangles), for the binding of imidazole to CcP(triLeu). The association rate constant, k_a^{app} , has a maximum value near pH 7 and was fit to Eq. 4 of the text where both ionizable groups affect the rate constant. k_a^{app} increases from a value of $2.2 \pm 0.4 \text{ M}^{-1} \text{ s}^{-1}$ at low pH to $98 \pm 165 \text{ M}^{-1} \text{ s}^{-1}$ at intermediate pH, then decreases at high pH to an undetermined value that is less than $25 \text{ M}^{-1} \text{ s}^{-1}$. The two apparent pK_a values converge to the same value of 7.3. The slow rate constant, k_{max} , has a small pH dependence that can be fit by an equation representing a single ionization. k_{max} increases from a value of $0.032 \pm 0.003 \text{ s}^{-1}$ at low pH to $0.077 \pm 0.011 \text{ s}^{-1}$ at high pH with an apparent pK_{aI} of 6.7 ± 0.4 . k_d^{app} is essentially independent of pH with an average value of $0.38 \pm 0.12 \text{ s}^{-1}$. Best-fit parameters are collected in Table 5. Experimental conditions: 0.10 M ionic strength buffers, 25 °C.

Figure 8.

Upper Panel: Titration of CcP(triVal) with imidazole at pH 7.0. The Soret band shifts from 409 to 412 nm with a 61% increase in absorptivity as the imidazole concentration increases from 0 to 50 mM. Lower Panel: Difference spectrum between CcP(triVal) in the presence and absence of 50 mM imidazole. Maximum and minimum peaks in the difference spectrum occur at 413 and 374 nm, respectively. The experiment was carried out in a double beam spectrophotometer with compensating imidazole in the reference cuvette to correct for the

imidazole absorbance. [CcP(triVal)] = 17.7 μ M, [imidazole] = 0 to 50 mM, 0.100 M ionic strength potassium phosphate buffer, pH 7.0, 25 °C.

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Figure 9. pH dependence of the negative logarithm of K_{D1} (solid circles) and K_{D2} (open circles) for binding of imidazole to CcP(triVal). The data were fit to Eq. 2 of the text using non-linear least squares regression. K_{D1} increases from 0.096 mM at high pH to 9.6 mM at low pH with an apparent pK_a of 5.7 ± 0.1 . K_{D2} increases from 6.8 mM at high pH to 380 mM at low pH with an apparent pK_a of 6.2 ± 0.3 . Best-fit parameters are collected in Table 2.

Figure 10.

pH dependence of the rate constants, k_a^{app} (Panel A), k_d^{app} (Panel B), and k_{max} (Panel C), for the fast (solid circles) and slow (open circles) imidazole binding phases to CcP(triVal). Panel A: Both association rate constants, k_a^{app} , are pH dependent and the data were fit to Eq. 4 of the text where only pK_{aI} affects the rate. For the fast phase of the reaction, k_a^{app} increases from a value of $5.4 \text{ M}^{-1} \text{ s}^{-1}$ at low pH to $311 \text{ M}^{-1} \text{ s}^{-1}$ at high pH, with a pK_{aI} of 6.2 ± 0.3 . For the slow phase of the reaction, k_a^{app} increases from a value of $1.3 \text{ M}^{-1} \text{ s}^{-1}$ at low pH to $227 \text{ M}^{-1} \text{ s}^{-1}$ at high pH, with a pK_{aI} of 7.6 ± 0.3 . Panel B: The dissociation rate

constant for the fast phase of imidazole binding is independent of pH, with an average value of $0.25 \pm 0.08 \text{ s}^{-1}$. k_d^{app} for the slow phase of the reaction is pH dependent, increasing from 0.026 s^{-1} at low pH to 0.073 s^{-1} at high pH with an apparent pK_a of 6.1 ± 0.5 . Panel C: k_{max} for both phases of the reaction is independent of pH with values of 1.5 s^{-1} for the fast phase and 0.17 s^{-1} for the slow phase. Bestfit parameters are collected in Table 5. Experimental conditions: 0.10 M ionic strength buffers, 25 °C.

Figure 11.

Upper Panel: Titration of CcP(triLeu) with 1-methylimidazole (MIM) at pH 7.0. The Soret band shifts from 404 to 414 nm with a 39% increase in absorptivity as the MIM concentration increases from 0 to 0.977 M. The dashed line shows the absorbance due to 0.977 M MIM. The experiments were carried out with a diode-array spectrophotometer and the spectra corrected for MIM absorbance by subtraction. Lower Panel: Difference spectrum between CcP(triLeu) in the presence and absence of 0.977 M MIM. Maximum and minimum peaks in the difference spectrum occur at 416 and 380 nm, respectively.

[CcP(triLeu)] = 7.13 μ M, [MIM] = 0 to 0.977 M, 0.100 M ionic strength potassium phosphate buffer, pH 7.0, 25 °C.

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Figure 12.

Upper Panel: Titration of CcP(triVal) with 4-nitroimidazole at pH 7.0. The Soret band shifts from 410 to 414 nm with a 34% increase in absorptivity as the 4-nitroimidazole concentration increases from 0 to 4.84 mM. The dashed line shows the absorbance due to 4.94 mM 4-nitroimidazole. The experiments were carried out with a diode-array spectrophotometer and the spectra corrected for 4-nitroimidazole absorbance by subtraction. Lower Panel: Difference spectrum between CcP(triVal) in the presence and absence of 4.84 mM 4-nitroimidazole. Maximum and minimum peaks in the difference spectrum occur at

420 and 382 nm, respectively. [CcP(triVal)] = 8.05 μ M, [4-nitroimidazole] = 0 to 4.84 mM, 0.100 M ionic strength potassium phosphate buffer, pH 7.0, 25 °C.

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Table 1

Equilibrium Dissociation Constants for Ligand Complexes of CcP, CcP Mutants, and metMyoglobin at pH 7^a

Enzyme	K_D (M)	Imidazole	1-Methylimidazole	4-Nitroimidazole
yCcP ^b	K_{D1}	3.3 ± 0.4	0.85 ± 0.11	~0.2
rCcP ^b	K_{D1}	4.6 ± 0.8	0.63 ± 0.04	~0.1
rCcP(H52L) ^b	K_{D1}	0.13 ± 0.04 (<46%)	1.1 ± 0.2	~0.03
	K_{D2}	9.5 ± 4.5 (>54%)		
CcP(TriAla)	K_{D1}	$(2.2 \pm 0.5) \times 10^{-4}$ (17%)	$(3.5 \pm 3.4) \times 10^{-4}$ (30%)	
	K_{D2}	$(1.2 \pm 0.1) \times 10^{-2}$ (83%)	$(1.1 \pm 0.1) \times 10^{-1}$ (70%)	$(1.6 \pm 1.1) \times 10^{-2}$
CcP(TriVal)	K_{D1}	$(8.3 \pm 0.5) \times 10^{-5}$ (68%)	$(1.6 \pm 0.4) \times 10^{-4}$ (77%)	$(5.6 \pm 0.2) \times 10^{-4}$
	K_{D2}	$(1.2 \pm 0.4) \times 10^{-2}$ (32%)	$(6.8 \pm 3.3) \times 10^{-3}$ (23%)	
CcP(TriLeu)	K_{D1}	$(6.0 \pm 0.4) \times 10^{-4}$ (78%)	-	
	K_{D2}	$(1.7 \pm 0.7) \times 10^{-2}$ (22%)	$(3.9 \pm 0.3) \times 10^{-2}$	$(3.5 \pm 1.3) \times 10^{-2}$
metMb ^{c,d}	K_{D1}	$(2.8 \pm 0.7) \times 10^{-2}$	$(8.7 \pm 1.0) \times 10^{-2}$	$(2.5 \pm 0.7) \times 10^{-3}$

^aThis work - pH 7.0, 0.10 M ionic strength potassium phosphate buffer,

^breference [1],

^creference [5],

^dreference [13].

Table 2

Parameters for the pH Dependence of the Equilibrium Dissociation Constants for High- and Low-Affinity Phases of Imidazole Binding to CcP Triple Mutants

Parameter ^a	CcP(triAla)	CcP(triVal)	CcP(triLeu)
K_{D1}^{acid} (mM)	>25	9.6 ± 5.5	120 ± 60
K_{D1}^{base} (mM)	0.13 ± 0.06	0.096 ± 0.007	0.22 ± 0.15
pK_{a1}	5.6 ± 0.5	5.7 ± 0.1	7.5 ± 0.4
K_{D2}^{acid} (mM)	570 ± 80	380 ± 390	160 ± 60
K_{D2}^{base} (mM)	6.4 ± 0.7	6.7 ± 2.3	9.4 ± 3.1
pK_{a2}	6.8 ± 0.1	6.2 ± 0.3	6.8 ± 0.4

^aParameters are defined in Eq. 2 of the text.

Table 3

Peak positions and extinction coefficients for CcP, CcP mutants, metMb, and their imidazole complexes at pH 7.0.

Protein ^a	protein band $\lambda(\epsilon)^b$	δ band $\lambda(\epsilon)^b$	Soret band $\lambda(\epsilon)^b$	visible bands $\lambda(\epsilon)^b$	
yCcP	282 (77)	374sh (57)	408 (98)	508 (11.3)	646 (3.3)
rCcP	281 (76)	374sh (60)	408 (101)	504 (11.2)	641 (3.5)
H52L	282 (86)	379sh (81)	404 (97)	511 (12.7)	651 (4.0)
triAla	277 (67)	356sh (51)	410 (104)	528 (11.4)	566sh (9.0)
triVal	279 (69)	356sh (42)	409 (81)	530 (9.2)	566 (6.7)
triLeu	277 (86)	380sh (69)	405 (89)	510sh (11.8)	640sh (4.0)
metMb	280 (39)	354sh (37)	409 (188)	503 (11.3)	581 (4.6)
yCcP-imidazole	-	-	412 (85)	542 (14.7)	570 (12.9)
rCcP-imidazole	-	260sh (31)	412 (109)	540 (10.7)	574sh (7.9)
triAla-imidazole	276 (69)	355 (40)	413 (143)	536 (15.8)	564sh (12.6)
triVal-imidazole	277 (68)	356 (28)	412 (138)	534 (12.1)	564sh (9.6)
triLeu-imidazole	277 (87)	354 (33)	412 (124)	532 (13.8)	564sh (11.0)
metMb-imidazole	280sh (36)	361 (32)	415 (146)	535 (12.9)	365sh (9.1)
yCcP-MIM	-	350 (29)	416 (132)	542 (13.5)	572 (12.0)
rCcP-MIM		346 (20)	420 (121)	544 (12.4)	574sh (10.1)
H52L-MIM		-	420 (133)	544 (13.7)	572sh (12.8)
triAla-MIM	276 (62)	356 (36)	414 (138)	536 (13.4)	562sh (11.2)
triVal-MIM	278 (64)	358 (23)	414 (117)	534 (10.8)	562sh (9.0)
triLeu-MIM	278 (79)	356 (28)	414(121)	534 (11.3)	562sh (9.4)
metMb-MIM	280sh (35)	357 (28)	417 (141)	534 (13.2)	562sh (9.8)
triVal-4NI	-	358 (37)	416 (110)	536 (10.7)	568sh (8.1)
metMb-4NI	271 (35)	359 (28)	416 (134)	537 (12.4)	568sh (8.6)

^a Abbreviations: MIM, 1-methylimidazole; 4NI, 4-nitroimidazole.

^b λ , wavelength in nm. ϵ , extinction coefficient in $\text{mM}^{-1} \text{cm}^{-1}$.

Table 4Kinetic Parameters for the Fast and Slow Phases of Imidazole Binding to the CcP Triple Mutants at pH 7^a

Protein	Kinetic Phase	k_a^{app} (M ⁻¹ s ⁻¹)	k_d^{app} (s ⁻¹)	k_{max} (s ⁻¹)
CcP(triAla)	Fast	80 ± 7	0.43 ± 0.04	-
CcP(triVal)	Fast	170 ± 7	0.32 ± 0.01	1.9 ± 0.1
CcP(triLeu)	Fast	36 ± 4	0.24 ± 0.03	-
CcP(triAla)	Slow	-	-	0.050 ± 0.009
CcP(triVal)	Slow	30 ± 10	0.050 ± 0.006	0.16 ± 0.08
CcP(triLeu)	Slow	-	-	0.070 ± 0.002

^a kinetic parameters defined in Eq. 6 of text.

Table 5Dependence of Apparent Rate Constants for Imidazole Binding to the CcP Triple Mutants^a

Protein	Kinetic Phase	Apparent Rate Constant	k^{acid}	k^{neut}	k^{base}	pK_{a1}	pK_{a2}
CcP(triAla)	fast	k_d^{app}	2.2 ± 0.4	99 ± 25		7.0 ± 0.2	
CcP(triLeu)	fast	k_d^{app}	2.2 ± 0.4	98 ± 165	0 ± 25	7.3 ± 0.9	7.3 ± 1.5
CcP(triVal)	fast	k_d^{app}	5.4 ± 16.2	311 ± 78		6.2 ± 0.3	
CcP(triVal)	slow	k_d^{app}	1.3 ± 0.7	227 ± 112		7.6 ± 0.3	
CcP(triAla)	fast	k_d^{app}		0.47 ± 0.10			
CcP(triLeu)	fast	k_d^{app}		0.38 ± 0.12			
CcP(triVal)	fast	k_d^{app}		0.25 ± 0.08			
CcP(triVal)	slow	k_d^{app}	0.026 ± 0.009	0.073 ± 0.011		6.1 ± 0.5	
CcP(triAla)	slow	k_{max}		0.032 ± 0.011			
CcP(triLeu)	slow	k_{max}	0.032 ± 0.003	0.077 ± 0.011		6.7 ± 0.4	
CcP(triVal)	fast	k_{max}		1.5 ± 0.3			
CcP(triVal)	slow	k_{max}		0.17 ± 0.04			

^a Apparent rate constants defined in Eq. 5 of the text, best-fit kinetic parameters defined in Eq. 4.