A Distal Histidine Mutant (H52Q) of Yeast Cytochrome *c* Peroxidase Catalyzes the Oxidation of H_2O_2 Instead of Its Reduction

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Abstract: A H52Q variant of yeast cytochrome *c* peroxidase (CcP), in which the distal histidine is replaced by glutamine, catalyzes *oxidation* of H_2O_2 instead of reduction. This redirection of catalytic action is detected by protein film voltammetry. In the presence of H_2O_2, wild-type CcP, adsorbed on a graphite electrode, shows a strong catalytic reduction wave commencing at about 0.8V (pH 5.4); by contrast, H52Q does not exhibit this activity but instead shows a catalytic *oxidation* current at potentials in the region of 0.9 V. The oxidation current is partly suppressed in the presence of tetranitromethane (a superoxide scavenger) and is not observed for other mutants studied, including H52A. The only significant structural change in the H52Q variant is that the Q-52 side chain occupies the space vacated by the H-52 imidazole; specifically, the N-ϵ atom that is believed to transfer a proton and induce O−O cleavage is replaced, to within 0.75 Å, by the carbamide-O. Thus, while the weakly basic amide functionality is unable to serve in the reorganization of bound H_2O_2, it is able to facilitate its oxidation, most obviously by serving as a H-bond acceptor to assist formation of a labile superoxide intermediate.

Introduction

In the catalytic cycle of heme peroxidases, reaction of the Fe(III) "resting state" with peroxide produces an intermediate called Compound I that contains Fe(IV)═O and a cation radical.[1] The first step is believed to be formation of an Fe(III)-peroxo species[2,3] that undergoes very rapid heterolytic O−O bond cleavage, assisted by a widely conserved distal histidine.[4,5] The peroxo species has proved difficult to characterize;[6] however, mutants lacking the distal histidine display greatly decreased peroxidase activity and much slower formation of Compound I.[7−9] In this paper, we show that the H52Q variant of yeast cytochrome *c* peroxidase (CcP), in which the distal histidine (His52) is replaced by glutamine, catalyzes *oxidation* of H_2O_2 *instead* of reduction. This redirection of catalytic action is detected and measured by protein film voltammetry, which addresses enzyme molecules adsorbed on an electrode surface.[10] Electron exchange between CcP and a pyrolytic graphite "edge" (PGE) electrode is fast;[11,12] this enables the catalytic properties to be probed over a range of potential, in this case under highly oxidizing conditions (>0.9 V vs SHE).

Methods

Wild-type (WT) and mutant (H52Q, H52A) forms of CcP (MKT) were obtained by similar procedures to those used previously.[13] Single crystals of H52Q were grown from 2-methyl-2,4-pentanediol (MPD) by vapor diffusion. Conditions for crystal growth, data collection, and analysis were as described before.[14] The crystal in space group $P2_12_12_1$ had unit cell dimensions $a = 105.04$ Å, $b = 74.54$ Å, and $c = 46.07$ Å, and gave diffraction to 1.98 Å (83% complete) with $R_{sym} = 0.049$, $I/\sigma_{I\ avg}$(2.0 to 8.0 Å) = 9.47 and $I/\sigma_{I\ avg}$(2.0 to 2.02 Å) = 0.6, and total number of reflections = 21 432. The structure was refined using XPLOR, which gave an $R_{cryst} = 0.202$ after two refinement cycles.[15]

Hydrogen peroxide (BDH Aristar) was diluted into buffer solutions and concentrations were determined by titration with standardized $KMnO_4$ on the day of experiments. Tetranitromethane and $K_2[IrCl_6]$, both from Aldrich, were used as received and solutions were made up by weight.

Voltammetry was performed with an Autolab potentiostat PGSTAT 10 or 20 (Eco Chemie, Utrecht, The Netherlands) equipped with an ECD module and controlled by GPES software (Eco Chemie). Data analysis was carried out using an in-house program: where necessary, the voltammetric data were smoothed using a fast Fourier transform

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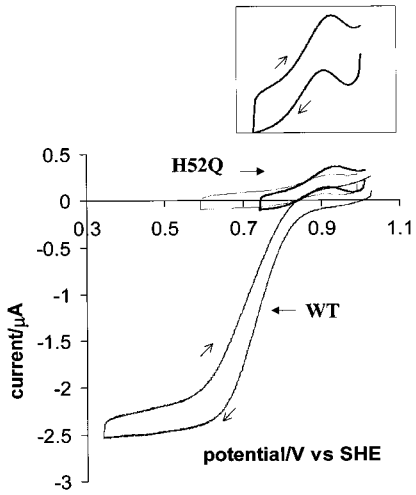

Figure 1. Protein film voltammograms of WT CcP and H52Q adsorbed on a PGE electrode and catalyzing redox transformations of H_2O_2 in opposite directions. In each case, the cell contains 160 mM H_2O_2 in 20 mM acetate (pH 5.4) at 0 °C, rotation rate 300 rpm, and scan rate 20 mV s^{-1}. Small arrows indicate cycle direction. The inset shows the H52Q voltammogram at expanded (×3) scale. The voltammetry of H52Q extended to more negative potential (light line) shows that this variant does not catalyze reduction of H_2O_2.

procedure, then the nonfaradaic background current was removed by subtracting a second-order polynomial function. A thermostated three-electrode glass cell or multipot cell, each with a Luggin sidearm housing a saturated calomel electrode (SCE), was used as described previously and housed in a Faraday cage to minimize electrical noise.[16] All potentials have been corrected to the standard hydrogen electrode (SHE) using $E_{SHE} = E_{SCE} + 241$ mV at 25 °C.[17] The rotating disk pyrolytic graphite "edge" (PGE) working electrode was constructed as described previously.[16]

Protein film voltammetry of cytochrome *c* peroxidase was carried out as reported earlier[11] except that enzyme was adsorbed by direct application (i.e. spreading with a fine pipet tip) of a 0.08−0.15 mM stock solution to the PGE electrode surface (area 0.03 cm^2), followed by 15 min immersion in the cell solution at open-circuit potential before starting cyclic voltammetry. Scans were initiated from either oxidizing or reducing limits without significant differences being observed.

Results and Discussion

Figure 1 shows the contrasting voltammetry displayed by wild-type (WT) CcP and the H52Q mutant. In each case, the enzymes are adsorbed on a rotating PGE disk electrode, which is immersed in buffer (pH 5.4) containing 160 μM H_2O_2 at 0 °C. As reported before,[11,12] WT CcP shows a strong catalytic wave due to reduction of H_2O_2, whereas H52Q does not exhibit this activity but instead shows a catalytic *oxidation* (positive) current. The form of the H52Q catalytic wave depends on H_2O_2 concentration, but is independent of scan direction and rate (1−50 mV s^{-1}). At low H_2O_2 levels, the wave is sigmoidal with an amplitude that is sensitive to electrode rotation rate—thus showing that catalysis is limited by substrate transport; whereas above 80 μM H_2O_2, the wave takes on a peak-like form (an oxidation peak is observed in both scan directions) that is insensitive to rotation rate. From the H_2O_2 dependence of current, K_M was determined as 0.45 ± 0.20 mM (pH 5.0−6.8)—the error arising from protein film instability at high potentials. In the absence of H_2O_2, WT CcP adsorbed on PGE gives a signal, consisting of sharp oxidation and reduction peaks, that is interpreted in terms of a reversible two-electron oxidation of the Fe(III) resting state. Integrations of this signal typically indicate monolayer coverage based on geometric electrode area. The same experiment carried out with H52Q produced instead weak, ill-defined voltammograms with larger peak separations; thus electrochemical redox transformations of H52Q are more sluggish than for WT CcP. Although it was not possible to measure, reliably, the electroactive coverage of H52Q, the attenuation of the WT signal that is observed when H52Q is included in the coating solution shows that the two variants compete for electrode sites,[12] suggesting, reasonably, that a monolayer of H52Q can also be formed. Consequently, assuming an enzyme coverage of 3×10^{-12} mol cm^{-2}, k_{cat} is approximately 30 s^{-1}.

Although the catalytic response appears small relative to the reduction wave given by the WT enzyme, it must be stressed that WT is very active (the second-order (specificity) constant $k_{cat}/K_M > 10^6$ M^{-1} s^{-1} at 4 °C):[11] thus the oxidation activity of H52Q is truly significant, with $k > 10^4$ M^{-1} s^{-1}. Between pH 4.6 and 7.5, the wave (peak potential) shifts from 0.95 to 0.80 V (gradient −52 mV) with little change in shape. At 0 °C, the expected slope for a H^+/e^- ratio of one is −54 mV.

The superoxide scavenger tetranitromethane (TNM)[18] attenuated the oxidation current (≥30% at 10 mM TNM, pH 5.4), but did not affect the reduction current with the WT enzyme. TNM had no effect on the "nonturnover" signals observed for either protein in the absence of H_2O_2, and thus does not alter the electroactive coverages. Tests were made to ascertain if catalytic H_2O_2 oxidation by H52Q could be observed by conventional methods, which required using a strong oxidant $[IrCl_6]^{2-}$ (reduction potential $E^{0\prime} = 0.95$ V)[19] as redox partner, in place of the electrode: stopped-flow studies revealed a small enhancement (up to 30%) in the rate of reduction of $[IrCl_6]^{2-}$ by H_2O_2 when H52Q (0.7−2.2 μM) was present. Additions of either $[IrCl_6]^{2-}$ or H_2O_2 to H52Q caused only a small shift of the Soret band (2 to 3 nm) with ϵ increasing slowly for $[IrCl_6]^{2-}$ but decreasing for H_2O_2 (>160 μM) and the 380 nm shoulder disappeared. By contrast, formation of Compound I in WT CcP is accompanied by a rapid 16 nm red shift of the Soret band and ϵ is increased relative to the ferric state. Other mutations at histidine-52 have been shown to catalyze reduction of peroxide at greatly decreased rates,[7] yet the H52Q mutant appears to be completely unreactive in this respect.

As shown in Figure 2, the only significant structural change in the H52Q variant is that glutamine-52 (Gln52) occupies the space vacated by the histidine, and is H-bonded to asparagine-82 (Asn82) via its amide-NH_2. The amide-O replaces, to within 0.75 Å, the imidazole N-ϵ atom that is believed to facilitate O−O bond cleavage by accepting the proximal proton and transferring it to the distal O-atom.[4]

The observation that H52Q yields a sizable oxidative current for H_2O_2 is unequivocal evidence that H_2O_2 is catalytically oxidized by this form of the enzyme. The fact that this activity is absent for other variants shows that oxidative catalysis occurs at the active site; i.e., H_2O_2 binds in the distal pocket, but the normal reduction pathway is blocked. The contrasting inactivity of H52Q toward reduction correlates with its inability to form Compound I from H_2O_2. Therefore, the H_2O_2 oxidation mechanism cannot involve peroxidatively generated Fe(IV)=O species, as proposed for the catalase-like activity of horseradish peroxidase.[20]

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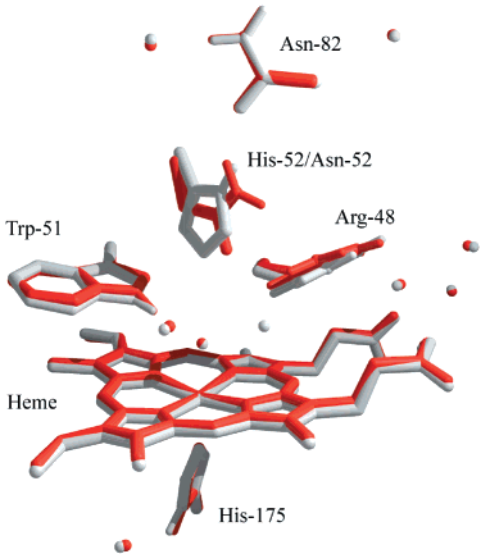
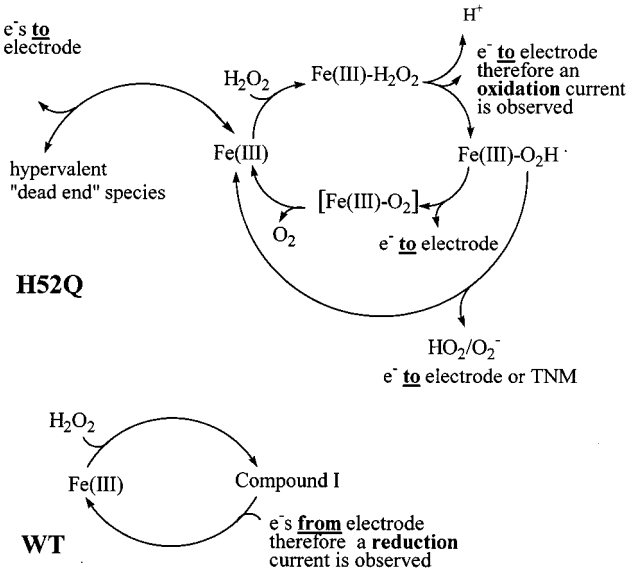


Figure 2. Active sites of WT CcP (white) and the H52Q mutant (red), from crystal structures obtained at 2.0 Å resolution.

Scheme 1. Proposed Catalytic Cycle for Oxidation of H_2O_2 by H52Q Cytochrome *c* Peroxidase, and Comparison with the normal Cycle for Reduction of H_2O_2 by WT Enzyme[a]



[a] The scheme also incorporates a possible explanation for why activity shuts down reversibly above a certain potential. This suggests formation of a "dead-end" state, perhaps through non-peroxidative electrochemical formation of Fe(IV)=O, or a similar species unable to bind H_2O_2. An alternative explanation, that the enzyme undergoes reversible reorientation on the electrode surface at high potential, cannot be ruled out.

The proposed catalytic cycle is shown in Scheme 1, a key feature being the formation of a Michaelis peroxo complex Fe(III)−H_2O_2. Normally, H_2O_2 is reduced; but here, faced with the difficulty of O−O cleavage in the absence of His52 and the "on-tap" availability of an electron sink (the electrode), the bound H_2O_2 is instead oxidized. This might occur via transient formation of a hypervalent active-site species (but not Fe(IV)=O). The H52A mutant displays negligible oxidative activity,

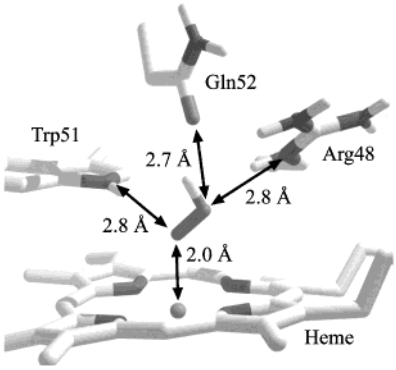


Figure 3. Modeling of bound peroxide (as HO_2^-) in the active site of the H52Q mutant of cytochrome *c* peroxidase. See text for description.

suggesting that the carbamide-O of Glu52 is required to bind H_2O_2 and/or facilitate proton removal. To assess this hypothesis, a bound peroxide was modeled into the structure. Figure 3 shows a hypothetical model for this interaction that was constructed by constraining one peroxo O-atom at 2.0 Å from the Fe and adjusting its orientation to optimize potential interactions with the distal cavity while avoiding steric conflicts. Several orientations are possible, but the one shown allows bound peroxide to donate a hydrogen bond to the carbonyl oxygen of Gln52, and to accept one from Arg48 and Trp51. It is clear that the carbonyl, and not the amide, of Gln52 is directed toward the heme due to a well-defined hydrogen bonding network involving Gln52, Asn82, Ser81, and Glu76. Thus, Gln52 is able to accept a hydrogen bond from peroxide, but is unable to transfer a proton from the proximal to distal peroxo oxygen atom as performed by His52 during heterolytic peroxy bond cleavage by the WT enzyme. The differing activities of the two enzymes must arise, at least partly, from the greatly decreased basicity of a carbonyl relative to imidazole. Attempts are underway to produce crystals of this reductively inert peroxide-bound state.

The high catalytic potential suggests that the rate-limiting step is the uphill oxidation of H_2O_2 to a superoxide species. These are considered in terms of the half-cell reactions given in eqs 1−3 and their reduction potentials.[21]

$$O_2^- + 2H^+ + e^- \rightleftharpoons H_2O_2 \qquad (1A)$$

$$HO_2 + H^+ + e^- \rightleftharpoons H_2O_2 \qquad (1B)$$

$$O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O_2 \qquad (2)$$

$$O_2 + e^- \rightleftharpoons O_2^- \qquad (3)$$

Between pH 5 and 7, $E^{0\prime}$ for the O_2^-/H_2O_2 couple (eqs 1A and 1B depending on pH; the p*K* of superoxide is around 5[22]) lies in the range 1.0 to 0.89 V, i.e. close to the catalytic peak potential, whereas $E^{0\prime}$ for the two-electron O_2/H_2O_2 couple (Equation 2) is much lower—in the range 0.39−0.28 V. The sensitivity to TNM shows further that superoxide is a labile intermediate, and the ratio $H^+/e^- = 1.0$ implied from the pH dependence of the catalytic peak potential suggests the importance of losing a single proton: possible interpretations are

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formation of O_2^- from bound HO_2^- (as modeled) or that superoxide is initially released as HO_2 from H_2O_2 (eq 1B). Superoxide is a good reductant; $E^{0\prime}$ for eq 3 is reported as −0.33 V at pH 7, so if released it will immediately be scavenged, either by the electrode or TNM.

Finally, the peak-like catalytic waveform, showing that the activity shuts down reversibly above a certain potential, suggests formation of a "dead-end" state that is unable to bind H_2O_2. One possibility is that this is due to nonperoxidative electrochemical formation of Fe(IV)═O derived from active site water molecules. This competing process is not expected to be fast, since experiments performed in the absence of substrate indicated that this ET reaction is more sluggish than for WT enzyme.[11] An alternative explanation is that the enzyme undergoes a reversible reorientation on the electrode surface at high potential.

This study provides a further demonstration of how voltammetric methods may be used to probe biologically relevant oxidation reactions occurring at high potentials in enzyme active sites. Detection of the redirection of redox activity of peroxide in H52Q is mechanistically informative and may have wider relevance in the study of enzymes that catalyze interconversion of oxygen species.[23]

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