Active-Site Mutations in Cytochrome c Peroxidase: A Critical Role for Histidine-52 in the Rate of Formation of Compound I

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Cytochrome *c* peroxidase active-site mutants have been constructed[1] in order to test a proposed mechanism[2] for the reaction between the enzyme and hydrogen peroxide to form compound I. The mechanism, based upon the crystallographic structure of cytochrome *c* peroxidase,[3] proposes that the distal histidine, His-52, functions initially as a base catalyst, promoting the ionization of hydrogen peroxide and coordination to the heme iron. A second residue, Arg-48, is in a position to interact with the distal oxygen atom of iron-coordinated peroxide. It is envisioned that Arg-48 facilitates the reaction by stabilizing the transition state as negative charge develops on the distal oxygen during the heterolytic cleavage of the oxygen–oxygen bond of the peroxide. The protonated form of His-52, generated in the initial interaction, can donate the proton to the newly formed hydroxide ion as it leaves the active site. Alternatively, both the distal histidine and the hydroxide can equilibrate with the buffered solution since the distal heme pocket is easily accessible to solvent.[3]

Of the active-site mutants to date, replacement of His-52 by a leucine residue has the greatest impact on the rate of compound I formation (Table I). The apparent bimolecular rate constant for compound I formation is decreased by 5 orders of magnitude in the mutant enzyme, CcP(H52L), compared to wild-type yeast enzyme, CcP(YST), or the parental form of the mutant enzyme expressed in *Escherichia coli*, CcP(MI). The value of the bimolecular rate constant for the reaction between CcP(H52L) and hydrogen peroxide is similar to the values observed for the reaction of hydrogen peroxide and metalloporphyrins[4] and between metmyoglobin and hydrogen peroxide.[5] Replacement of the distal histidine in cytochrome *c* peroxidase with an aliphatic residue has eliminated the rate enhancement normally shown by this enzyme over that of small, inorganic active-site model complexes or nonenzymatic heme proteins such as metmyoglobin, suggesting that His-52 is the key residue in accelerating the reaction.

The effect of replacing (the conserved) Arg-48 is not as dramatic. Elimination of the positive charge, by replacing Arg-48 with a leucine residue, CcP(R48L), decreases the rate by about 2 orders of magnitude. The rate remains some 3 orders of magnitude faster than the analogous reaction of peroxide with the metalloporphyrins or metmyoglobin,[4,5] even in the absence of a positive charge at position 48. It is also interesting to note that replacing Arg-48 with a lysine residue decreases the rate by only 2-fold, suggesting that the positively charged ϵ-amino side chain of Lys is nearly as effective as the guanidinium side chain in promoting peroxide heterolysis. The present results suggest that conservation of the distal Arg may be more important in stabilizing compound I than in promoting heterolysis. A positive charge is not an absolute requirement for rapid reaction with hydrogen peroxide, as already noted by the lack of a positively charged residue near the distal site of catalase.[6]

Table I. Apparent Rate Constants for the Reaction between Cytochrome *c* Peroxidase Mutants and Hydrogen Peroxide and for the Endogenous Reduction of the Fe(IV) Heme in Compound I[a]

protein	k_1[b] (M^{-1} s^{-1})	k_{decay}[c] (s^{-1})
CcP(YST)	$(3.9 \pm 0.5) \times 10^7$	$(3.0 \pm 0.3) \times 10^{-5}$
CcP(MI)	$(3.2 \pm 0.5) \times 10^7$	$\sim 6 \times 10^{-5}$
CcP(W191F)	$(5.7 \pm 0.4) \times 10^7$	$(6.8 \pm 0.7) \times 10^{-5}$
CcP(D235N)	$(6.0 \pm 0.4) \times 10^6$	$(3.3 \pm 0.4) \times 10^{-3}$[d]
CcP(R48K)	$(1.3 \pm 0.2) \times 10^7$	$(1.1 \pm 0.1) \times 10^{-2}$
CcP(R48L)	$(1.5 \pm 0.1) \times 10^5$	$(3.0 \pm 0.1) \times 10^{-2}$
CcP(H52L)	$(2.9 \pm 0.6) \times 10^2$	$(2.5 \pm 0.4) \times 10^{-3}$

[a] All data were acquired in 10 mM potassium phosphate buffer, pH 6, with KNO_3 to adjust the ionic strength to 0.1 M, 25 °C. [b] Apparent bimolecular rate constant for the reaction between hydrogen peroxide and enzyme to form compound I. [c] Rate of absorbance decrease at 424 nm due to the endogenous reduction of the Fe(IV) site in compound I to Fe(III). [d] The decay rate of this mutant is very dependent upon the nature of the buffer and is significantly more stable in 0.1 M phosphate buffer, pH 6.

We have previously determined the hydrogen peroxide reaction rate for two proximal-side mutants, CcP(W191F)[7] and CcP(D235N).[8] Trp-191 is the residue which is oxidized to a free-radical state in CcP compound I.[7,9] Replacement of Trp-191 with a phenylalanine residue prevents the amino acid radical from forming. CcP(W191F) reacts almost twice as fast with hydrogen peroxide compared to CcP(MI), forming an oxyferryl porphyrin π-cation radical intermediate[7] similar to that of HRP compound I.

Asp-235 is buried within the core of CcP and is hydrogen bonded to the proximal histidine, His-175, and to Trp-191.[3] This interaction imparts imidazolate-like character to His-175,[10] which should make the oxygen–oxygen bond of peroxide more labile as well as stabilize the higher oxidation states of CcP.[11] Replacement of Asp-235 with an asparagine residue dramatically alters the coordination of the ferric iron.[8] At pH 6, ferric CcP(D235N) exists primarily as a hydroxide form rather than as the pentacoordinate form characteristic of ferric CcP. Despite the occupation of the sixth coordination site by hydroxide, the reaction of CcP(D235N) is decreased only 4-fold relative to the parent. If one assumes a dissociative mechanism for peroxide heterolysis and at least 80% occupancy of the sixth coordination site by the hydroxide ligand, the rate of reaction of the putative pentacoordinate form of CcP(D235N) with peroxide must be at least as fast as that for the native pentacoordinate form of CcP(MI). This leads to the conclusion that the negative charge at position 235, and the resulting imidazolate-like character of the proximal histidine, had little effect on the rate of compound I formation although it does seem to stabilize compound I. The endogenous reduction of the Fe(IV) group in compound I to the Fe(III) state is 2 orders of magnitude faster in CcP(D235N) when compared to CcP(MI). Modification of the distal side residues at positions 48 and 52 also significantly increases the endogenous reduction of compound I. This may be due to the disruption of the hydrogen-bonded network of solvent and polar residues in the distal heme pocket.

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We have carried out initial characterization of CcP mutants which include changes at all of the proximal and distal sites that have been suggested to play a role in the facilitation of oxygen–oxygen bond cleavage and compound I formation (with the exception of the proximal histidine).[2,11] Replacement of the distal histidine residue has the most profound effect, reducing the reaction rate by 5 orders of magnitude. Before we can conclude that this change is due to the loss of catalysis by His-52, we must eliminate such trivial causes for enzyme inactivation as major reorganization of the enzyme structure or changes which block access of hydrogen peroxide to the active site. However, crystallographic structures of CcP(W51F), CcP(W191F), and CcP(D235N)[12] show that only small, localized structural perturbations occur in these active-site mutants, and we expect the same to be true for CcP(H52L). Nevertheless, a definitive conclusion must await the structure of CcP(H52L). Crystallographic studies of CcP(H52L) are underway.

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Observation of Triplet-State Electron Spin Resonance in Oxidized C_{60}

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The formation, electronic structure, stability, and reactivity of both the reduced and the oxidized C_{60} fullerene molecules are of intense current interest.[1,2] Triplet-state ESR spectra have been observed for reduced C_{60} produced by electrochemical[3] and photochemical[4] methods. ESR spectra of $S = {}^1/_2$ radicals have been reported for C_{60} dissolved in concentrated and fuming sulfuric acids[5] and in Magic Acid.[6] We now report the observation of triplet-state ESR for C_{60} chemically oxidized in fuming sulfuric acid.

Dissolution of column-purified C_{60} (>99.9% pure) in fuming sulfuric acid (27% free SO_3) at 263 K with 5% SO_2FCl added to suppress the freezing temperature results in the formation of a green solution. Electron spin echo detected ESR (ESE-ESR) spectra[7] of oxidized C_{60} recorded at 100 K are shown in Figure 1. The spectra comprise an overlap of several ESR signals. The most striking feature is the classic Pake absorption pattern characteristic for a randomly oriented triplet ($S = 1$) spin system.[8]

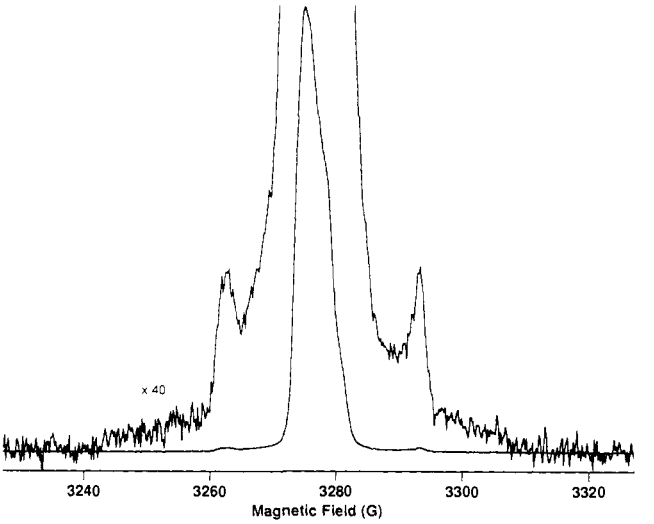

Figure 1. Electron spin echo detected ESR spectrum of C_{60} in fuming sulfuric acid. Experimental conditions: microwave frequency, 9.1850 GHz; pulse widths, 0.40 and 0.80 μs; spin echo interpulse delay, 0.45 μs; pulse sequence repetition rate, 2.5 kHz; temperature, 100 K; sweep width, 100 G; sweep size, 512 points.

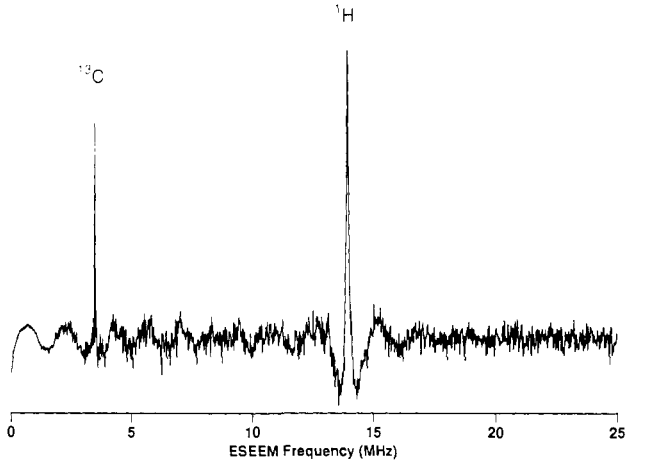

Figure 2. Cosine Fourier transform of the electron spin echo envelope (ESEEM) of C_{60} in fuming sulfuric acid. Experimental conditions: microwave frequency, 9.185 GHz; pulse widths for stimulated echo, 0.02 μs; delay between pulses one and two: 0.17 μs; delay between pulses two and three, from 0.05 to 20.51 μs; pulse sequence repetition rate, 667 Hz; temperature, 90 K; magnetic field, 3276.1 G.

The zero-field parameter, $|D|$, can be measured directly from the singularities in the EPR spectrum[9] and yields $|D| = 31$ G.

A strong peak at the ^{13}C Larmor frequency was observed in electron spin echo envelope modulation[10] (ESEEM) spectra (Figure 2) of the same sample. Since the C_{60} molecule is the only source of carbon in the solution, the observation of the ^{13}C peak confirms that the $S = 1$ ESR signal arises from the oxidized C_{60} molecule. The second peak in the ESEEM spectrum is at the proton Larmor frequency. It is attributed to the dipolar coupling of the oxidized C_{60} radical ion with protons on solvent molecules.

Since ESEEM spectroscopy is most sensitive to weak nuclear hyperfine couplings, pulsed ENDOR[11,12] spectroscopy was used to search for evidence of larger ^{13}C and ^{1}H hyperfine couplings. As shown in the pulsed ENDOR spectra[13] of Figure 3, only the

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