Detection of an Oxyferryl Porphyrin π-Cation-Radical Intermediate in the Reaction between Hydrogen Peroxide and a Mutant Yeast Cytochrome *c* Peroxidase. Evidence for Tryptophan-191 Involvement in the Radical Site of Compound I†

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ABSTRACT: Peroxide oxidation of a mutant cytochrome *c* peroxidase, in which Trp-191 has been replaced by Phe through site-directed mutagenesis, produces an oxidized intermediate whose stable UV/visible absorption spectrum is very similar to that of compound I of the native yeast enzyme. This spectrum is characteristic of an oxyferryl, Fe(IV), heme. Stopped-flow studies reveal that the reaction between the mutant enzyme and hydrogen peroxide is biphasic with the transient formation of an intermediate whose absorption spectrum is quite distinct from that of either the native ferric enzyme or the final product. Rapid spectral scanning of the intermediate provides a spectrum characteristic of an oxyferryl porphyrin π-cation-radical species. At pH 6, 100 mM ionic strength, and 25 °C, the rate constant for formation of the oxyferryl π-cation radical has a lower limit of 6×10^7 M^{-1} s^{-1} and the rate of conversion of the transient intermediate to the final oxidized product is 51 ± 4 s^{-1}. Evidence is presented indicating that Trp-191 either is the site of the radical in CcP compound I or is intimately involved in formation of the radical.

One of the major differences between yeast cytochrome *c* peroxidase (CcP)[1] and other members of the peroxidase family of enzymes is the nature of the initial intermediate formed by stoichiometric oxidation of the native ferric state of the enzyme with hydrogen peroxide. Most peroxidases, including horseradish peroxidase (HRP), form a species called compound I, in which the native ferric heme group has undergone 2-equiv oxidation to an oxyferryl, Fe(IV), porphyrin π-cation-radical state (Blumberg et al., 1968; Dolphin et al., 1971; Hewson & Hager, 1979; Rutter et al., 1983). This oxidized species is characterized by a diminished absorptivity in the Soret region and an increase in absorptivity between 600 and 700 nm (Dolphin et al., 1971). EPR and Mössbauer studies indicate strong magnetic coupling between the porphyrin radical and the Fe(IV) group (Rutter et al., 1983). In contrast, CcP compound I has a UV/visible spectrum (Yonetani, 1965) similar to that of HRP compound II (Hewson & Hager, 1979) in spite of the fact that HRP compound II is oxidized 1 equiv above the ferric state while CcP compound I is oxidized 2 equiv above the ferric state (Coulson et al., 1971). The UV/visible spectra of both CcP compound I and HRP compound II are characteristic of the oxyferryl, Fe(IV), heme group. The second oxidizing equivalent in CcP compound I exists as an amino acid free radical (Yonetani et al., 1966). Mössbauer, EPR, and ENDOR studies indicate that the protein-based free radical and the Fe(IV) group are not magnetically coupled, suggesting that the two oxidized sites are far apart in CcP compound I (Lang et al., 1976; Hoffman et al., 1981).

Despite considerable effort, the radical site (or sites) in CcP compound I has not been identified. The easily oxidized aromatic and sulfur-containing amino acids are the most likely candidates. The single cysteine residue in CcP has been ruled out since modification of this residue has no effect on the catalytic properties of CcP (Yonetani, 1976). Upon discovery of the free-radical-like EPR spectrum, Yonetani et al. (1966) suggested an aromatic amino acid was oxidized on the basis of the changes in the UV spectrum during oxidation of CcP to compound I (Yonetani, 1965). Subsequently, oxidation of tryptophan and tyrosine residues was shown to occur during the endogenous reduction of the oxyferryl and radical sites in compound I (Coulson & Yonetani, 1972). Trp-51 became a prime candidate when the three-dimensional structure of CcP was determined. The indole ring of Trp-51 is parallel and in van der Waals contact with the distal heme face (Finzel et al., 1984). However, spectroscopic evidence for a nitrogen-based radical is conflicting, and Met-172 has been considered an alternative candidate for the radical (Hoffman et al., 1979, 1981; Lerch et al., 1981; Myers & Palmer, 1985).

A more direct approach to the identification of the radical is through site-directed mutagenesis.[2] In the simplest case, if the residue that gives rise to the radical signal is replaced by a residue that cannot be oxidized, then oxidation of the mutant CcP by hydrogen peroxide should produce an intermediate that looks like horseradish peroxidase compound I, an oxyferryl porphyrin π-cation-radical intermediate.

Both Met-172 (Goodin et al., 1986) and Trp-51 (Fishel et al., 1987; Goodin et al., 1987) have been replaced by nonox-

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[1] Abbreviations: CcP, bakers' yeast cytochrome *c* peroxidase; CcP(MI), cytochrome *c* peroxidase expressed in *E. coli*; CcP(MI,F191), cytochrome *c* peroxidase in which Trp-191 has been replaced by Phe by site-directed mutagenesis; HRP, horseradish peroxidase.

[2] A caveat in mutagenesis studies should be noted. While a negative result is conclusive (i.e., if an amino acid substitution causes no change in radical signal, the original residue is not involved), a positive result is not. Various amino acid substitutions could perturb the structure of the protein, indirectly altering the radical site.

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idizable amino acids, and all mutants retain catalytic activity. Mutants at both sites retain the axially symmetric EPR signal at 10 K characteristic of CcP compound I when oxidized by peroxide (Goodin et al., 1987) and have the typical CcP compound I UV/visible spectrum, leading to the conclusion that neither of these two residues is the primary radical site in CcP compound I.

Comparison of the crystallographic structures of CcP compound I with that of the native enzyme reveals small structural perturbations in a cluster of amino acid residues including Trp-191, Met-230, and Met-231 (Edwards et al., 1987). Oxidation of Met-230 or Met-231 to the sulfoxide produces an enzyme with no detectable reaction with hydrogen peroxide (Kim & Erman, 1988). Replacement of Trp-191 with a phenylalanine residue, producing the mutant CcP(MI,F191), decreases the catalytic activity by a factor of ~3000, although addition of hydrogen peroxide produces an oxidized intermediate with an UV/visible spectrum similar to that of yeast CcP compound I (with lowered absorptivity between 580 and 640 nm) and an isotropic EPR signal, lacking the broad "wings", at 89 K (Mauro et al., 1988). Recent EPR studies (Scholes et al., 1989) demonstrate that the axially symmetric EPR signal is absent at 4.2 K in the peroxide-oxidized intermediate of CcP(MI,F191). This is the expected EPR result if Trp-191 is required for generation of the radical site in the native enzyme. However, the UV/visible spectrum of peroxide-oxidized CcP(MI,F191) is not that of the expected oxyferryl porphyrin π-cation radical. In this report we show that the oxyferryl porphyrin π-cation radical is indeed formed during the reaction between CcP(MI,F191) and hydrogen peroxide but is subsequently reduced to give the more stable oxyferryl heme group, oxidized a single equivalent above the ferric state. Apparently, the second oxidizing equivalent is very unstable in peroxide-oxidized CcP(MI,F191), generating the observed isotropic radical EPR signal which decays very rapidly ($t_{1/2}$ ~1 min).

The present study, demonstrating formation of the oxyferryl porphyrin π-cation radical during the oxidation of CcP(MI,F191) by hydrogen peroxide, along with the 4.2 K EPR studies, indicating the absence of the axially symmetric EPR signal (Scholes et al., 1989), provides strong evidence that Trp-191 either is the site of the stoichiometric radical in yeast CcP compound I or is intimately involved in formation of the radical.

Materials and Methods

Twice crystallized CcP(MI,F191) was prepared as previously described (Fishel et al., 1987; Mauro et al., 1988). Concentrations of the enzyme were determined spectroscopically at pH 6 by using a millimolar absorptivity of 109 at 408 nm (Mauro et al., 1988). Hydrogen peroxide (30%, J. T. Baker Chemical Co.) was standardized with cerium(IV) sulfate according to Kolthoff and Belcher (1957). All studies were carried out at pH 6.0 in a 10 mM potassium phosphate buffer with sufficient KNO_3 to adjust the ionic strength to 0.100 M.

Spectra were obtained with a Cary 219 UV/visible recording spectrophotometer at a scan rate of 1 nm/s. Stopped-flow experiments were performed with a Hi-Tech Scientific PQ/SF-53 stopped-flow spectrofluorometer equipped with an MG-3000 spectrascan accessory. The spectrascan accessory allowed acquisition of spectra during the stopped-flow runs at a rate of 90 nm/ms. The observation path length was 1 cm. Reactant reservoirs, drive syringes, mixer, and observation chambers were thermostated at 25 °C with a circulating water bath. The reactions were studied under pseudo-first-order conditions with the hydrogen peroxide in excess over the enzyme.

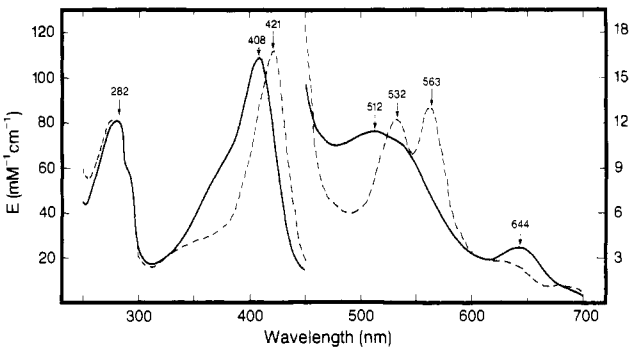

FIGURE 1: UV/visible spectra of CcP(MI,F191) and CcP(MI,F191) compound I. The Soret maxima for CcP(MI,F191) (solid line) and CcP(MI,F191) compound I (dashed line) are at 408 and 421 nm, respectively. At isosbestic point occurs at 414 nm.

Results

Titration of CcP(MI,F191) with Hydrogen Peroxide. At pH 6, the UV/visible spectrum of CcP(MI,F191) is very similar to that of CcP (Yonetani, 1965) and CcP(MI) (Mauro et al., 1988). Addition of stoichiometric amounts of hydrogen peroxide oxidizes the native ferric state of the enzyme to a higher oxidation state called compound I (Figure 1). The spectrum of CcP(MI,F191) compound I is nearly identical with that of CcP compound I (Yonetani, 1965) and CcP(MI) compound I (Mauro et al., 1988) with a Soret maximum at 421 nm and maxima in the visible region at 532 and 563 nm. The only significant difference is about a 30% decrease in the intensity of the shoulder observed between 580 and 640 nm for CcP(MI,F191) compound I in comparison to that of CcP(MI) compound I (Mauro et al., 1988).

A spectrophotometric titration of CcP(MI,F191) with hydrogen peroxide, monitored at 424 nm, shows a 1:1 stoichiometry for the CcP(MI,F191)/hydrogen peroxide reaction. At pH 6, the spectral characteristics and hydrogen peroxide titration behavior of CcP(MI,F191) are essentially identical with those of CcP and CcP(MI).

Stopped-Flow Studies. In contrast to the spectral characteristics and hydrogen peroxide titration behavior, the stopped-flow traces of the CcP(MI,F191)/hydrogen peroxide reaction were very different from those of CcP and CcP(MI). At 424 nm, both CcP and CcP(MI) show increases in absorbance upon being mixed with hydrogen peroxide as compound I is formed. However, with CcP(MI,F191) there was an initial decrease in absorbance followed by the expected increase in absorbance at 424 nm when the enzyme is mixed with hydrogen peroxide. The unexpected decrease in absorbance suggested rapid formation of an intermediate with an absorptivity smaller than that of the native ferric state of the enzyme.

To confirm the presence of a spectrally distinct intermediate in the reaction between CcP(MI,F191) and hydrogen peroxide to form compound I, we carried out stopped-flow experiments at 414 nm, the isosbestic point between CcP(MI,F191) and CcP(MI,F191) compound I (Figure 1). We observed substantial changes in absorbance upon mixing CcP(MI,F191) and hydrogen peroxide when the reaction was monitored at 414 nm (Figure 2). A large decrease in absorbance occurred during the dead time of our stopped-flow instrument. We were able to observe a small portion of the decrease in absorbance, followed by a slower increase in absorbance, up to the level corresponding to that of both CcP(MI,F191) and compound I.

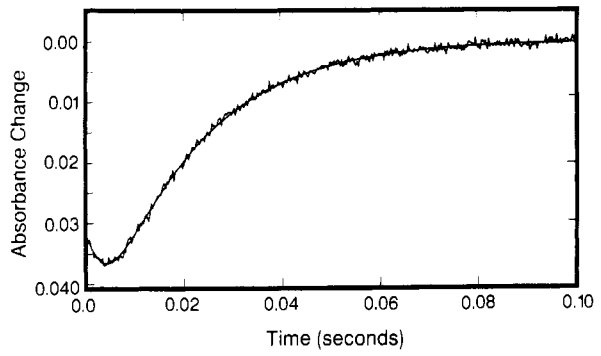

FIGURE 2: Change in absorbance after CcP(MI,F191) and hydrogen peroxide are mixed at 414 nm. The concentrations of enzyme and hydrogen peroxide were 0.94 and 4.93 μM, respectively. The solid line is a theoretical fit to eq 1 of the text with values of 254 and 54.3 s^{-1} for k_A and k_B, respectively.

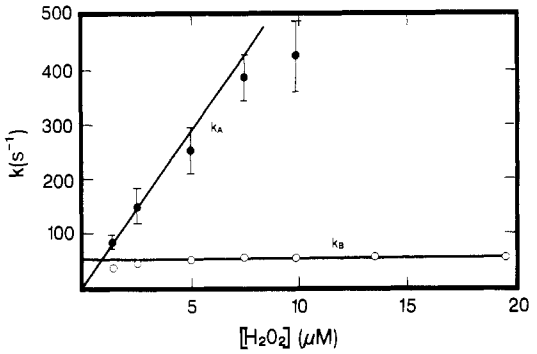

FIGURE 3: Plots of k_A and k_B as a function of hydrogen peroxide concentration. k_A (solid circles) increases linearly with hydrogen peroxide while k_B (open circles) is essentially independent of hydrogen peroxide.

Values of the observed rate constants for reaction with H_2O_2 were obtained by fitting the time rate of change of the absorbance to a two-exponential empirical equation

$$\Delta A = \Delta A_A \exp(-k_A t) + \Delta A_B \exp(-k_B t) + C \quad (1)$$

A nonlinear least-squares regression analysis was used to determine values of the rate constants and preexponential factors. Since so much of the fast reaction, which causes the decrease in absorbance at 414 nm, occurs during the dead time of the apparatus, it is clear that the mixing process itself is influencing the apparent rate for this step. Our fitting procedure most likely underestimates the true value of k_A, and the reported values should be considered lower limits. Values of k_B could be accurately determined, and the reported values are the mean of at least five determinations and have an average standard deviation of 12%.

Concentration Dependence of the Observed Rate Constants. The rates of the two observed reactions were determined as a function of hydrogen peroxide concentration between 1 and 20 μM, with the results shown in Figure 3. The value of k_A depends upon the hydrogen peroxide concentration while k_B is essentially independent of the hydrogen peroxide concentration. Mechanisms to account for the concentration dependences are discussed in a later section.

Rapid Spectral Scanning. The observation of two rate processes and the absorbance changes at 414 nm, the isosbestic point between native CcP(MI,F191) and its oxidized intermediate compound I, indicated that a transient intermediate occurs during the reaction of CcP(MI,F191) and hydrogen peroxide. In order to determine the spectrum of the intermediate, rapid spectral scanning experiments were performed, with the results shown in Figure 4. Curve 1 is the spectrum of CcP(MI,F191) determined on the stopped-flow instrument

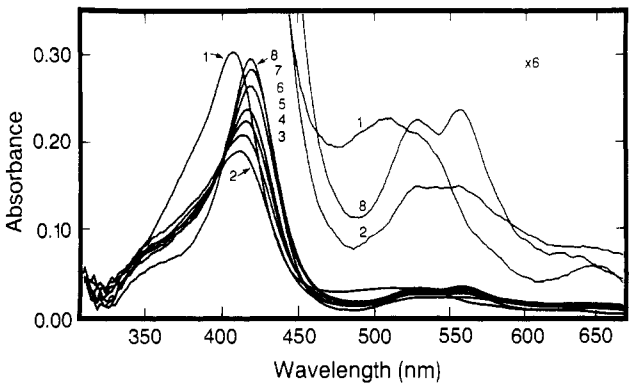

FIGURE 4: Rapid spectral scanning of the reaction between CcP(MI,F191) and hydrogen peroxide. Curve 1 is the spectrum of 3.53 μM CcP(MI,F191) mixed with buffer in the stopped-flow apparatus. Curves 2–8 are spectra of the reaction mixture at various times after 3.53 μM CcP(MI,F191) and 19.6 μM hydrogen peroxide are mixed. The times after mixing hydrogen peroxide and enzyme (determined at 400 nm) for curves 2–8 are 6, 8, 12, 16, 24, 32, and 856 ms, respectively. Each spectrum is the result of a separate stopped-flow experiment. The visible portion of curves 1, 2, and 8 is also shown expanded 6-fold.

with the spectrascan accessory. Curve 2 is the spectrum of CcP(MI,F191) 6 ms after being mixed with excess hydrogen peroxide. There is a substantial decrease in absorptivity throughout the Soret region. Curves 3–8 show the increase in absorbance as CcP(MI,F191) compound I is formed, with its Soret maximum at 421 nm.

Discussion

Evidence for the Formation of a Porphyrin π-Cation-Radical Intermediate. The very large multiphasic spectral changes that occur during the reaction between CcP(MI,F191) and hydrogen peroxide clearly demonstrate the presence of a transient intermediate during the formation of CcP(MI,F191) compound I. The transient intermediate dominates the spectrum shown in curve 2 of Figure 4. This spectrum is very similar to that of HRP compound I (Blumberg et al., 1968; Hewson & Hager, 1979). The dramatic decrease in the Soret absorptivity of the transient intermediate, in comparison to that of the ferric heme state of the enzyme, is consistent with the removal of an electron from the porphyrin π system generating a porphyrin π-cation radical (Dolphin et al., 1971). Because the spectral characteristics of the transient intermediate in the CcP(MI,F191)/hydrogen peroxide reaction are so similar to that of HRP compound I and to the stable π-cation-radical states of synthetic metalloporphyrins (Dolphin et al., 1971), we conclude, by analogy, that the transient intermediate is an oxyferryl porphyrin π-cation-radical form of CcP(MI,F191).

Mechanism for CcP(MI,F191) Compound I Formation. The time rate of change of the absorbance during the reaction between CcP(MI,F191) and hydrogen peroxide can be explained by the mechanism

$$\text{CcP(MI,F191)} + H_2O_2 \xrightarrow{k_1} I_A \xrightarrow{k_2} I_B \quad (2)$$

The species denoted I_A is the newly discovered oxyferryl porphyrin π-cation-radical form of compound I. Species I_B is the spectroscopically stable form of oxidized CcP(MI,F191) (Mauro et al., 1988). Under pseudo-first-order conditions, where hydrogen peroxide is in excess, k_A is equal to $k_1[H_2O_2]$ and k_B is equal to k_2. Values of k_1 and k_2 were evaluated from the data in Figure 4 by nonlinear least-squares regression analysis. Since the values of k_A are considered lower limits, the lower limit for k_1 was determined to be 60 μM^{-1} s^{-1}. The

best fit value for k_2 is 51 ± 4 s^{-1}.

There are no large structural perturbations in crystallized CcP(MI,F191) (J. Wang, unpublished X-ray crystallographic observations), but resonance Raman spectroscopy suggests some differences in CcP(MI,F191) in comparison to CcP and CcP(MI) (Smulevich et al., 1988). Between pH 5.4 and 7, the ν_3 band for CcP(MI,F191) is centered at 1492 cm^{-1}, indicative of 5-coordinate high-spin Fe(III), but the band is broad with appreciable intensity at 1475 and 1504 cm^{-1}. The latter positions indicate 6-coordinate high-spin and low-spin Fe(III) forms, respectively. The ν_3 bands for CcP and CcP(MI) show less of the 6-coordinate forms at neutral pH. In spite of the larger fraction of 6-coordinate species in CcP(MI,F191), the lower limit for the bimolecular rate constant for the hydrogen peroxide reaction, k_1, is almost twice as large as for CcP under comparable conditions (Loo & Erman, 1975) and CcP(MI) (L. Vitello and J. Erman, unpublished observations). This may be the result of eliminating the Trp-191/Asp-235 hydrogen bond which can in turn influence the Asp-235/His-175 and His-175/Fe(III) interactions. Smulevich et al. (1988) argue that elimination of the Asp-235/His-175 interaction allows more flexibility in the position of the iron atom relative to the plane of the porphyrin and favors 6-coordinate iron. This argument, along with our kinetic results, suggests that displacement of the coordinated ligand (water most likely) is not rate limiting but that movement of the iron relative to the porphyrin plane could influence the rate-limiting step in the initial hydrogen peroxide/enzyme interaction.

Chance et al. (1967) have presented evidence for a transient intermediate in the reaction between yeast CcP and hydrogen peroxide and have speculated that this intermediate was equivalent to HRP compound I. The present studies indicate that the formation of the porphyrin π-cation radical does occur in CcP(MI,F191) and supports the hypothesis that such a sequence can occur in yeast CcP.

We believe that the formation of the oxyferryl porphyrin π-cation radical during the reaction between CcP(MI,F191) and hydrogen peroxide, as demonstrated in this report, and the absence of the axially symmetric EPR signal at 4.2 K in hydrogen peroxide oxidized CcP(MI,F191) (Scholes et al., 1989) offer strong evidence that Trp-191 is either the radical site or a major component of a radical-site cluster as proposed by Edwards et al. (1987).

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