Fluorescence Investigation of Yeast Cytochrome *c* Peroxidase Oxidation by H_2O_2 and Enzyme Activities of the Oxidized Enzyme†

Ted Fox,‡ George Tsaprailis, and Ann M. English*

Department of Chemistry and Biochemistry, Concordia University, 1455 de Maisonneuve Boulevard West, Montreal, Quebec, Canada H3G 1M8

Received July 23, 1993; Revised Manuscript Received October 12, 1993•

ABSTRACT: The role of tryptophan residues as endogenous electron donors in cytochrome *c* peroxidase (CCP) was examined by protein steady-state fluorescence. Compound I and more highly oxidized forms of CCP were formed by adding 1, 3, and 10 equiv of H_2O_2 to 5 μM protein at pH 7.0 in the absence of *exogenous* reducing substrates. Addition of native CCP to 8 M urea at pH 1.5 relieved heme quenching, and compound I exhibited 90 ± 4% fluorescence relative to unoxidized CCP, consistent with the loss of 0.7 ± 0.2 tryptophan and the assignment of the primary radical site to Trp191. CCP oxidized with 10-fold excess H_2O_2 exhibited 65 ± 1% relative fluorescence, indicating loss of 2.4 ± 0.1 tryptophans. Compound I and the higher oxidized forms of CCP spontaneously decayed to ferric CCP species over ~24 h with the loss of ~0.5 *additional* tryptophan in each case. The 24-h decay product of compound I exhibited 73% activity, 74% H_2O_2 titer, and titration led to the further oxidation of ~0.6 tryptophan. However, no further tryptophan oxidation was observed on titration of the 24-h decay products of samples initially oxidized with 3 and 10 equiv of H_2O_2. These samples exhibited 58 and 18% H_2O_2 titer, and 47 and 16% activity, respectively, which shows that radical formation on Trp191 is *not* required for activity. The fluorescence decrease with time paralleled the decrease in activity of H_2O_2-oxidized CCP using both ferrocytochrome *c* and ferrocyanide as substrates, indicating that tryptophan and activity loss occurred on similar time scales. Since CCP reduced 3 and 10 equiv of H_2O_2 within 5 and 20 min, respectively, but fluorescence and activity loss increased slightly over 24 h, charge migration must occur in the polypeptide during decay, giving rise to 7–33% dimer formation along with the loss of the 0.5 additional tryptophan.

Binding of H_2O_2 to ferric heme peroxidases results in heterolytic cleavage of the O–O bond and the formation of oxidized enzyme intermediates. The two- and one-electron oxidized intermediates are termed compound I and compound II, respectively. To regenerate ferric peroxidase, $P(Fe^{III})$, electrons are provided by donor substrates, and the enzymic cycle is as follows:

$$P(Fe^{III}) + H_2O_2 \rightarrow \text{compound I } (X^{\bullet}Fe^{IV}{=}O) + H_2O \quad (1)$$

$$\text{compound I} + 1e^- \rightarrow \text{compound II} \quad (2)$$

$$\text{compound II} + 1e^- \rightarrow P(Fe^{III}) + H_2O \quad (3)$$

In compound I, the oxidizing equivalents are stored as the oxyferryl state (Fe^{IV}=O) of the heme iron, and an organic radical, X•. In most heme peroxidases, X• is a porphyrin π-cation radical (Dawson, 1988).

Cytochrome *c* peroxidase (CCP),[1] a hemoprotein found in yeast mitochondria, catalyzes the reduction of H_2O_2 by ferrocytochrome *c*. A porphyrin radical is not observed in the stable compound I of CCP, so an alternate site for X• had long been sought. Several residues had been implicated as possible radical sites, including Trp51 (Finzel et al., 1984; Poulos & Finzel, 1984); Hori et al., 1985; Goodin et al., 1987; Fishel et al., 1987; Scholes et al., 1989), Trp191 (Hori et al., 1985; Scholes et al., 1989; Erman et al., 1989), Met172 (Finzel et al., 1984; Goodin et al., 1986), and Met230 and Met231 (Edwards et al., 1987; Hoffman et al., 1979). Recently, ENDOR measurements on isotopically labeled CCP (Sivaraja et al., 1989) identified a tryptophan residue as the radical site. Trp191 appeared to be the most likely candidate since the EPR of compound I of the CCP(W191F) mutant does not exhibit the broad signal characteristic of native CCP (Scholes et al., 1989; Fishel et al., 1991). Also, unlike wild-type CCP, this mutant transiently shows a porphyrin radical on reaction with peroxide (Erman et al., 1989). Trp51, which is only 4 Å from the heme, was eliminated as the radical site since Trp51 → Phe mutation had no effect on the EPR or ENDOR spectra of compound I (Scholes et al., 1989).

In the absence of an oxidizable substrate, the Fe^{IV}=O and radical (X•) sites of compound I decay slowly to give a product which has an absorption spectrum similar to native ferric CCP (Erman & Yonetani, 1975a). The decay product has lost the 2 oxidizing equiv of compound I but retained ~75% activity (Erman & Yonetani, 1975b). Amino acid analysis showed loss of 0.2 tryptophan, 0.5 tyrosine, and 0.5 phenylalanine at pH 7 (Coulson & Yonetani, 1972), suggesting that the 2 latter residues are the major endogenous donors. Moreover, it has been reported that CCP can reduce up to 10 equiv of H_2O_2 without detectable O_2 formation by further oxidation of its amino acid residues (Erman & Yonetani, 1975b). Amino acid analysis indicated that the decay product following oxidation of CCP with 10-fold excess H_2O_2 (10:1 product) has lost 3.4 tyrosines, 1.5 phenylalanines, and 0.6 tryptophan

† This research was supported by a grant from NSERC (Canada) to A.M.E.

* Author to whom correspondence should be addressed.

‡ Present address: Vertex Pharmaceuticals, Inc., 40 Allston St., Cambridge, MA 02139.

• Abstract published in *Advance ACS Abstracts*, December 1, 1993.

[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate), CCP, cytochrome *c* peroxidase (EC 1.11.1.5); compound I, two-electron oxidized species formed when 1 equiv of H_2O_2 reacts with CCP; compound II, species formed on one-electron reduction of compound I; ENDOR, electron nuclear double resonance; EPR, electron spin resonance; hemin, ferric protoporphyrin IX heme; NATA, *N*-acetyltryptophanamide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/94/0433-186$04.50/0

at pH 7. Peroxide at millimolar concentrations readily oxidizes methionine residues in proteins but not aromatic residues (Neumann, 1967); hence, oxidation of the latter in CCP at low H_2O_2 concentrations ($\leq 50\ \mu M$) must be mediated by the heme.

CCP is the first enzyme known to use a stable tryptophan radical during its turnover (Prince & George, 1990). Since protein emission is dominated by tryptophan, the present study explores the use of steady-state fluorescence measurements to probe the involvement of tryptophan residues in the catalytic cycle of CCP and the loss of these residues in H_2O_2-oxidized CCP. Efficient quenching by the heme results in a fluorescence quantum yield for CCP of only 7% relative to the tryptophan standard NATA (Fox et al., 1993), but in 8 M urea at pH 1.5, heme quenching is relieved due to heme dissociation from the polypeptide. Since the seven tryptophans in *denatured* CCP are exposed to an aqueous environment, they are expected to possess the same emission quantum yields, allowing fluorescence loss to be equated with tryptophan loss.

However, excitation of tryptophan is also accompanied by tyrosine excitation. Tyrosine and tryptophan have emission maxima at ~303 and ~350 nm in water, respectively, and in the absence of tyrosine to tryptophan energy transfer, the tyrosine contribution to the emission intensity at 350 nm will be negligible. Following correction for inner-filter effects, 1 μM CCP (which contains 7 μM tryptophan) and 7 μM NATA exhibited the same fluorescence intensity in 8 M urea at pH 1.5. This indicates that tyrosine to tryptophan energy transfer, which has an R_0 of ~14 Å (Lakowicz, 1983), approaches zero efficiency in *denatured* CCP. Denatured alcohol dehydrogenase, which possesses 6 tryptophans and 14 tyrosines, also showed zero energy transfer while the efficiency was 70% in the native state (Saito et al., 1981; Lakowicz, 1983). Hence, although CCP possesses 14 tyrosines, which are also likely endogenous donors, tyrosine loss is not expected to affect the emission intensity at 350 nm.

To determine the extent of electron donation from tryptophan residues in compound I and the 3:1 and 10:1 products, fluorescence intensities at 350 nm were measured at various time intervals following oxidation. Enzyme activities, using both ferrocytochrome *c* and ferrocyanide as reducing substrates, were also measured at similar time intervals just prior to denaturation to compare rates of fluorescence loss with activity loss. CCP denaturation in 8 M urea at pH 1.5 occurs within the mixing time (2–3 s) which should inhibit protein radical migration *after* sample addition to urea. To probe radical migration to surface residues in oxidized CCP, the extent of peroxide-induced *intermolecular* cross-linking was determined by SDS–PAGE. After 24 h, compound I and CCP oxidized with up to 10:1 H_2O_2 give rise to stable decay products (Erman & Yonetani, 1975a). Activities, H_2O_2 titers, and further loss of tryptophan in the 24-h decay products were examined to ascertain whether Trp191 was necessary for activity since CCP(W191F) has only 0.03% activity (Mauro, 1988). Amino acid analysis of compound I and the 10:1 product was reexamined here to compare tryptophan loss with fluorescence loss.

EXPERIMENTAL PROCEDURES

Horse heart cytochrome *c* (type III), *N*-acetyltryptophanamide (NATA), and guanidinium chloride were obtained from Sigma; ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)] was purchased from Boehringer Mannheim as the diammonium salt, and analytical grade urea was purchased from Anachemia. All chemicals were used without further purification. Distilled water (specific resistance 18 MΩ cm) was prepared by a Barnstead Nanopure system. SDS–PAGE was performed on 7.5% acrylamide gels using a Mini Protean II Electrophoresis Dual Slab Cell and silver staining (Bio-Rad). Densitometer tracings of the gels were obtained on a FisherBiotech transmission densitometer. Fluorescence and absorption measurements were carried out on a Shimadzu Model RF 5000 spectrofluorometer and a Hewlett Packard 8451A diode-array spectrophotometer, respectively.

H_2O_2 Oxidation of CCP. A 5 μM CCP stock solution was prepared in 0.1 M sodium phosphate buffer (pH 7.0) assuming $\epsilon_{408} = 98\ mM^{-1}\ cm^{-1}$ (Yonetani & Anni, 1987). Stock H_2O_2 solutions (0.2–2 mM) were prepared in the same buffer and added to 5 μM CCP to give the desired H_2O_2:CCP molar ratios. Formation of oxyferryl heme was followed spectrophotometrically at 424 nm (Erman & Yonetani, 1975a).

Enzyme Activity of Oxidized CCP. Activity measurements using ferrocytochrome *c* (Yonetani & Ray, 1965) or ferrocyanide (Spangler, 1984; Jordi & Erman, 1974) as reducing substrates were performed by following substrate oxidation spectrophotometrically (ferrocytochrome *c*, $\Delta\epsilon_{550} = 18\ mM^{-1}\ cm^{-1}$; ferrocyanide, $\Delta\epsilon_{420} = 1.0\ mM^{-1}\ cm^{-1}$). Activities of H_2O_2-oxidized CCP species are reported relative to native CCP maintained under the same conditions.

H_2O_2 Turnover and H_2O_2 Titer of Oxidized CCP. ABTS (20 μM) oxidation as monitored at 724 nm was used to determine the extent of H_2O_2 turnover by CCP (5 μM) at various times. The 24-h decay products were titrated with H_2O_2, and the absorbance change at 424 nm was monitored to determine the extent of oxyferryl heme formation.

Relative Fluorescence. At various time intervals (15 s–24 h) after the addition of H_2O_2 to 5 μM CCP in 0.1 M phosphate buffer (pH 7.0), protein samples were diluted with urea to give a final concentration of 1 μM CCP in 8 M urea at pH 1.5. Following 280-nm excitation, fluorescence intensities were measured at 350 nm, which is the emission maximum of CCP in urea. The Raman peak of water was substracted from all emission spectra, and inner-filter effects were corrected using the formula (Lakowicz, 1983):

$$F = F_o\ \text{antilog}[(A_{ex} + A_{em})/2] \quad (4)$$

where F is the corrected fluorescence intensity, F_o is the observed intensity, and A_{ex} and A_{em} are the absorbances at the excitation and emission wavelengths, respectively. Relative fluorescence intensities (% F) were calculated assuming % F = 100 for unoxidized CCP maintained under the same conditions.

SDS–PAGE of Protein Samples. Cross-linking of CCP following addition of 1, 3, and 10 equiv of H_2O_2 was investigated by SDS–PAGE according to the published procedure (Nielsen & Reynolds, 1978). At various time intervals (15 s–24 h) following addition of H_2O_2 to 5 μM CCP in 0.1 M phosphate buffer (pH 7.0), 10 μL of enzyme was added to 40 μL of 10% (w/v) SDS. Samples were allowed to stand in SDS for 30 min and heated (95 °C) for 5 min prior to loading 0.85 μg of protein per sample on the gels which were developed using silver staining. The percent monomer present in oxidized CCP relative to unoxidized CCP was estimated by integration of the densitometer tracings of the gels.

Amino Acid Analysis. Prior to amino acid analysis, 5 μM native CCP, compound I, and the 10:1 product were maintained at 22 °C for 24 h. Both base (8 N KOH at 110 °C for 14 h) and acid (4 N methanesulfonic acid at 110 °C for 14 h) hydrolyses of the proteins were carried out, and the

fig1

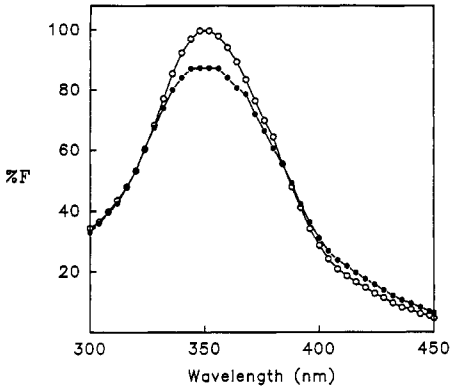

FIGURE 1: Relative fluorescence of 1 μM CCP (open circles) and compound I (closed circles) in 8 M urea at 22 °C on excitation at 280 nm. Slits, 5 nm; scan rate, 114 nm/min. Data points are the corrected (eq 4) relative fluorescence intensities, %F.

amino acid content of the products was determined using standard procedures (Spackman et al., 1958; Darbre, 1986).

RESULTS

Relative Fluorescence. Quenching of native CCP emission by the heme is eliminated in 8 M urea at pH 1.5. The fluorescence increase upon denaturation, which is observed within the mixing time (2–3 s), is accompanied by a red-shift in the emission maximum to 350 nm, characteristic of tryptophan exposure to an aqueous environment (Lakowicz, 1983). Furthermore, after correcting for inner filter effects (eq 4), 1 μM CCP, which contains 7 μM tryptophan, and 7 μM NATA exhibited the same fluorescence intensity in 8 M urea at pH 1.5. This is consistent with zero tyrosine to tryptophan energy transfer and zero heme quenching under these conditions. In 8 M urea *at pH 7*, residual quenching by the heme was still observed, and the sharp Soret absorption (not shown) indicated that the heme is still coordinated to the CCP polypeptide. It is also of interest that tyrosine emission at 303 nm was not observed for denatured CCP on excitation at 275 nm, the tyrosine absorption maximum.

In phosphate buffer (pH 7.0), both CCP and compound I exhibit emission maxima at 325 ± 2 nm, and the 109% relative fluorescence of the latter can be accounted for by red-shifting of the Soret on compound I formation (Fox et al., 1993). In contrast, denatured compound I has 90 ± 4% relative fluorescence in 8 M urea (Figure 1), indicating loss of emission from 0.7 ± 0.2 of the 7 tryptophans in the protein. Addition of 1 reducing equiv (ascorbate) before denaturation increased the relative fluorescence to 98 ± 1%, and the addition of a second reducing equivalent resulted in no further increase in fluorescence. Presumably the amino acid radical (X•) is reduced before the heme since 1 reducing equiv did not shift the Soret from the 420-nm maximum for compound I, but a shift to 408 nm, characteristic of ferric CCP, was observed on addition of 2 reducing equiv. When 10-fold excess H_2O_2 was added to CCP 15 s prior to denaturation, the relative fluorescence in 8 M urea was 86 ± 3%, indicating that only 1.0 ± 0.2 tryptophan was oxidized in this short time interval. However, within 5 min, the relative fluorescence had decreased to 75%, revealing loss of a second tryptophan whereas the relative fluorescence of compound I remained at 90%.

Figure 2A summarizes the fluorescence intensities at 350 nm following denaturation of CCP at various intervals after addition of 1, 3, and 10 equiv of H_2O_2. Assuming an equal contribution from each of the seven tryptophans, the decrease in fluorescence was converted to the number of tryptophans

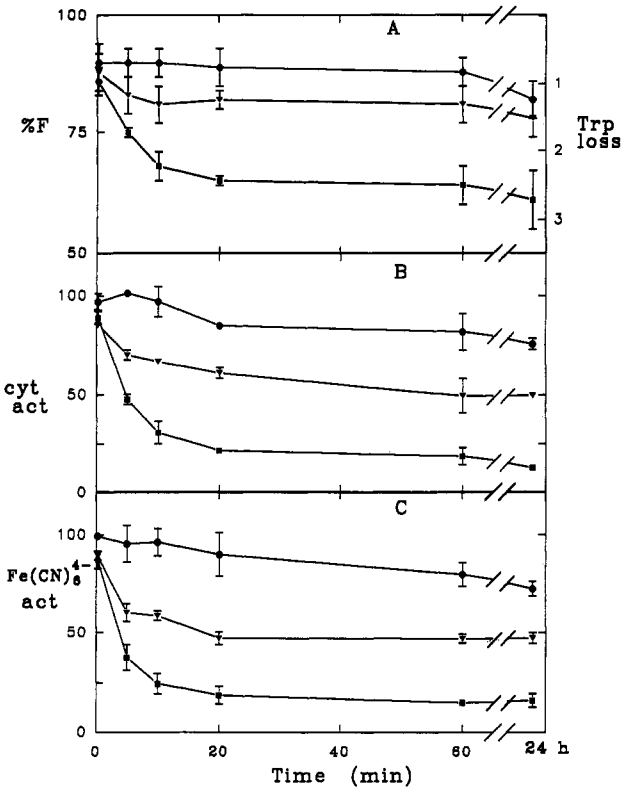

FIGURE 2: (A) Fluorescence intensities at 350 nm of 1 μM H_2O_2-oxidized CCP in 8 M urea, pH 1.5. CCP (5 μM) was reacted with H_2O_2 in 0.1 M phosphate buffer, pH 7.0, and diluted with urea at various times after addition of H_2O_2. 1:1 H_2O_2/CCP (compound I), circles; 3:1 H_2O_2/CCP, triangles; and 10:1 H_2O_2/CCP, squares. Data points are the averages of four measurements. See Figure 1 for experimental conditions. (B) Ferrocytochrome *c* and (C) ferrocyanide oxidizing activities in 0.1 M phosphate buffer, pH 7.0, relative to native CCP of the samples in (A) just prior to denaturation.

lost. Clearly this number *increases* with time and the amount of H_2O_2 added, and ~3 tryptophans are lost in the 10:1 24-h decay product. Essentially identical results (data not shown) were obtained in 6.4 M guanidinium chloride at pH 7.0, but samples in 8 M urea at pH 7.0 were less fluorescent due to heme association with the polypeptide under these conditions.

Enzyme Activity of Oxidized CCP. Activities using ferrocytochrome *c* and ferrocyanide as reducing substrates are shown in panels B and C, respectively, of Figure 2. Within 15 s of oxidation, ≥85% activity was observed for all samples with both substrates, but the activities of the 3:1 and 10:1 products rapidly decreased in ≤20 min. Activities of all samples further decreased over 24 h, and the residual activities using both substrates are listed in Table 1.

H_2O_2 Turnover and H_2O_2 Titer of Oxidized CCP. Compound I formed within the mixing time, and addition of ABTS to the CCP–peroxide samples showed that the turnover at pH 7.0 of 3 and 10 equiv of H_2O_2 was complete within 5 and 20 min, respectively. Using the relative absorbance change at 424 nm (the maximum in the ferric and oxyferryl CCP difference spectrum), the 24-h decay products were titrated with H_2O_2 to determine the extent of their ability to re-form oxyferryl heme, and the H_2O_2 titers are also listed in Table 1. $\Delta\epsilon_{424}$ for the decay products is the same as that for native CCP as reported previously for titrations at pH 8 (Erman & Yonetani, 1975).

Soret Absorption. Ferric CCP and compound I exhibit Soret maxima at 408 and 420 nm, respectively (Figure 3A). The broad Soret absorption bands observed immediately following addition of ferric CCP, compound I, and free hemin

Table 1: Properties of the 24-h Decay Products of H_2O_2-Oxidized CCP[a]

property (%)[b]	1 equiv of H_2O_2	3 equiv of H_2O_2	10 equiv of H_2O_2
H_2O_2 titer[c]	74 ± 5	58 ± 2	18 ± 0.4
monomer[d]	93	78	77
cyt *c* act.[e]	73 ± 3	47 ± 2	16 ± 3
$Fe(CN)_6^{4-}$ act.[e]	76 ± 3	50 ± 2	13 ± 2
Trp loss[f]	~0.6	0	0

[a] CCP (5 μM) was oxidized with 1, 3, and 10 equiv of H_2O_2 in 0.1 M phosphate buffer, pH 7.0, and properties were measured 24 h after oxidation. [b] Relative to unoxidized CCP maintained under the same conditions. [c] From H_2O_2 titrations (see text). [d] From Table 2. [e] Activities from Figure 2B,C. [f] Tryptophan loss at 15 s following addition of 1 equiv of H_2O_2 to the decay products. The loss was estimated from the change in fluorescence of the decay products in 8 M urea (pH 1.5) before and after the addition of H_2O_2 (see text).

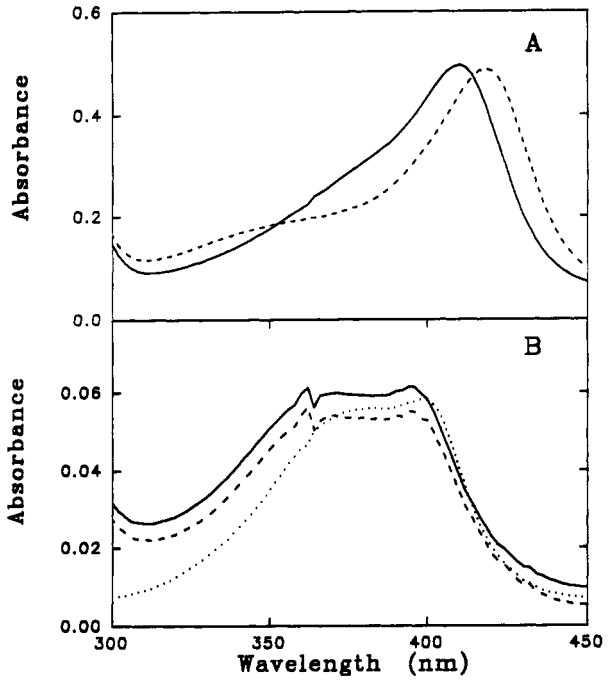

FIGURE 3: Soret absorption of ferric CCP (solid line) and compound I (dashed line). (A) 5 μM protein in 0.1 M phosphate, pH 7.0; (B) 1 μM protein in 8 M urea, pH 1.5. The dotted line in (B) is the absorption of 0.44 μM hemin.

Table 2: Percent Monomer in H_2O_2-Oxidized CCP Samples[a]

	% monomer[b]		
time	1 equiv of H_2O_2	3 equiv of H_2O_2	10 equiv of H_2O_2
15 s	100	93	95
5 min	100	88	83
1 h	100	89	79
24 h	93	78	77

[a] CCP (5 μM) was oxidized with 1, 3, and 10 equiv of H_2O_2 in 0.1 M phosphate buffer, pH 7.0, and denatured at the times indicated. [b] Percent monomer relative to unoxidized CCP as estimated by integration of densitometer tracings of the gels.

to 8 M urea at pH 1.5 are essentially identical (Figure 3B). This suggests that the three hemes are in a similar environment and have the same iron coordination and oxidation states. Hence, the protein-bound ferric and oxyferryl hemes are released, and the latter is rapidly reduced upon denaturation at pH 1.5.

SDS–Page of Protein Samples. Table 2 summarizes the percent monomer in H_2O_2-oxidized CCP at pH 7.0. Gels of compound I and its 24-h decay product showed that essentially all the protein is monomeric whereas the monomeric content of the 3:1 and 10:1 24-h decay products has decreased to ~78%. These results are in marked contrast to those reported previously for CCP oxidized at *pH 4.5* using ≥10-fold higher protein concentrations (Spangler, 1984; Spangler & Erman, 1986) where 75, 25, and 0% monomer were observed for the 1:1, 3:1, and 10:1 products, respectively. The significantly higher monomer content observed here is probably due in part to the lower protein concentrations used in this study, but may also arise from the pH dependence of the decay pathways since oxidation of CCP of low and high pH produced different products (Erman & Yonetani, 1975b).

Amino Acid Analysis of Oxidized CCP. The amino acid compositions of CCP and the compound I decay product showed no significant differences, but the 10:1 decay product had 2.4 ± 0.3 less tyrosines and 1.7 ± 0.2 less tryptophans than native CCP, in reasonable agreement with the loss of 3.4 tyrosines and 0.6 tryptophan (alkaline hydrolysis) reported previously at pH 7 (Coulson & Yonetani, 1972). Thus, compared to the fluorescence measurements, amino acid analysis underestimates tryptophan loss in compound I and the higher oxidized forms of CCP, which may be due to the problems encountered in tryptophan determination (Darbre, 1986).

DISCUSSION

Compound I exhibits 90 ± 4% fluorescence at 350 nm relative to unoxidized CCP in 8 M urea at pH 1.5. This corresponds to the loss of 0.7 ± 0.2 tryptophan and supports the assignment of the radical site (X•, eq 1) to a tryptophan residue (Sivaraja et al., 1989). The uncertainty in tryptophan loss may be due to reduction of the radical by trace impurities in the urea so that the value of 0.7 may in fact be a lower limit. Upon denaturation, compound II (eq 2), which stores its single oxidizing equivalent as Fe^{IV}=O at pH 7 (Coulson et al., 1971), shows 98 ± 1% relative fluorescence; hence, most of the observed fluorescence loss in compound I is due to radical formation and *not* to the rapid reduction of the Fe^{IV}=O heme in urea (Figure 3B). Reduction of compound II to ferric CCP before denaturation had not further effect on the fluorescence in 8 M urea.

CCP species oxidized with 3 and 10 equiv of H_2O_2 prior to denaturation are significantly less fluorescent than compound I (Figure 2A), and loss of 2.8 ± 0.4 tryptophans is observed for the 10:1 24-h decay product. An examination of the 3-D structure of CCP shows a ring of aromatic residues on the proximal side that could channel charge to the heme (Edwards et al., 1987). There are three tyrosines (187, 229, 236) within 5 Å of Trp191 and a pair of tryptophans (211, 223) within 6–9 Å (Figure 4). Oxidation of these residues would be consistent with the fluorescence data (Figure 2A) and the amino acid analyses which indicated loss of ~3 tyrosines over 24 h in the 10:1 decay product. Figure 2A shows that tryptophan loss is greatest within the 20 min following H_2O_2 addition to CCP, which mirrors the rate of peroxide turnover by CCP; 3 equiv is reduced per mole of enzyme in ≤5 min and 10 equiv in ≤20 min. The further loss of ~0.5 tryptophan indicates that charge migration occurs in the polypeptide over the period from 1 to 24 h.

Decay of compound I over 24 h clearly does not restore tryptophan fluorescence to 100% (Figure 2A); thus, a stable oxidation product of Trp191 or a neighboring tryptophan must be formed during the decay process. Since the 24-h decay product of compound I exhibits 73% ferrocytochrome *c* oxidizing activity, irreversible oxidation of Trp191 would be surprising considering that CCP(W191F) has only 0.03%

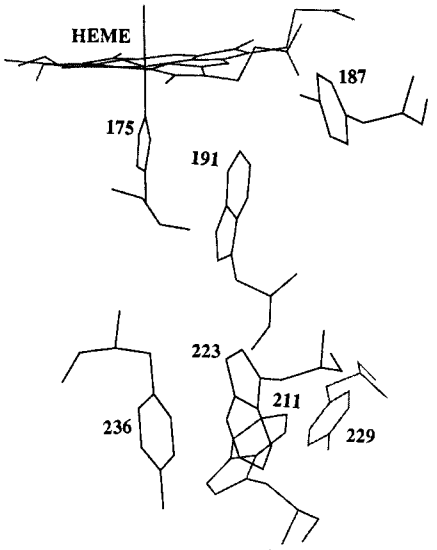

FIGURE 4: Locations of the three tryptophans (191, 211, 223) and three tyrosines (187, 229, 236) relative to the heme and proximal His 175 of CCP. This molecular graphics image was adapted from Figure 6 of Edwards et al. (1987) and produced using the MidasPlus software system from the Computer Graphics Laboratory, University of California, San Francisco (Ferrin et al., 1988).

activity (Mauro et al., 1988). However, titration with H_2O_2 regenerates the spectrum of compound I and leads to the immediate loss of 0.6 tryptophan (Table 1), suggesting that the second oxidizing equivalent ($X^{\bullet}$, eq 1) may also be located mainly on Trp191 in the decay product. H_2O_2 titration of the 3:1 and 10:1 24-h decay products, on the other hand, results in no further oxidation of tryptophan, precluding radical formation on Trp191. Since these species retain 47 and 16% ferrocytochrome *c* oxidizing activity, respectively (Table 1), radical formation on Trp191 must *not* be necessary for high ferrocytochrome *c* turnover; this is contrary to expectation considering the negligible ferrocytochrome *c* oxidizing activity of CCP(W191F). Clearly, a stopped-flow investigation of the reaction between H_2O_2 and the decay products would be of interest, particularly since transient porphyrin radical formation was observed for CCP(W191F) (Erman et al., 1989).

The fluorescence loss in H_2O_2-oxidized CCP parallels the dependence of activity on H_2O_2:CCP ratios and time (Figure 2), indicating that tryptophan loss and activity loss occur on similar time scales. Within 15 s of H_2O_2 addition, ≥85% activity is observed for all the samples, but, like the fluorescence, the activity of the 3:1 and 10:1 products decreases significantly over the 20-min period required for reduction of 10 equiv of H_2O_2 by the enzyme. In all samples, activity loss continued over 24 h, as well as the loss of monomer due to protein cross-linking (Table 1). This is further evidence of charge migration in the polypeptide, which results in the loss of ~0.5 additional tryptophan. Both the ferrocytochrome *c* and ferrocyanide oxidizing activities of the 24-h decay products are the same, within experimental error, as the H_2O_2 titers (Table 1), which was found previously at pH 8.0 (Erman & Yonetani, 1975b); thus, activity loss at pH 7.0 is due to the reduced capacity of the enzyme to react with H_2O_2.

The fluorescence and activity data presented here on oxidized CCP underscore the important role played by tryptophan residues in the redox chemistry of the enzyme. The loss of activity and H_2O_2 titer with protein fluorescence indicates that tryptophan residues are important in controlling the reactivity of CCP toward H_2O_2. Since ~3 tryptophan residues are lost during the endogenous reduction of peroxide and amino acid analyses reveal loss of ~3 tyrosines, electron donation to H_2O_2 via the heme from the ring of aromatic residues shown in Figure 4 is a possibility as suggested previously (Edwards et al., 1987). HPLC peptide mapping coupled with fluorescence detection and electrospray mass spectrometry are being used to unravel the specific aromatic residues that act as endogenous donors to H_2O_2, and the role of Trp191 in CCP activity.

This study demonstrates that steady-state fluorescence measurements under denaturing conditions are a valuable probe of tryptophan loss in oxidized CCP. The lack of significant fluorescence loss in CCP(W191F) compound I (G. Tsaprailis, unpublished results) provides convincing evidence for the validity of this approach. Amino acid analysis is a much more time-consuming, and much less accurate and less sensitive, method for tryptophan determination than fluorescence intensity measurements. Furthermore, the variation in EPR signals following mutation of residues around Trp191 has complicated the assignment of aromatic radicals in CCP mutants (Fishel et al., 1991; Goodin & McRee, 1993; Houseman et al., 1993); hence, fluorescence measurements will be useful in distinguishing between tyrosine and tryptophan radicals in these mutants.

ACKNOWLEDGMENT

We thank Bernard F. Gibbs of the Biotechnology Research Institute (Montreal) for the amino acid analysis.

REFERENCES

Coulson, A. F. W., & Yonetani, T. (1972) *Biochem. Biophys. Res. Commun. 49*, 391.
Coulson, A. F. W., Erman, J. E., & Yonetani, T. (1971) *J. Biol. Chem. 246*, 917.
Darbe, A. (1986) in *Practical Protein Chemistry—A Handbook*, p 239, Wiley, New York.
Dawson, J. H. (1989) *Science 240*, 433.
Edwards, S. L., Xuong, N. H., Hamlin, R. C., & Kraut, J. (1987) *Biochemistry 26*, 1503.
Erman, J. E., & Yonetani, T. (1975a) *Biochim. Biophys. Acta 393*, 350.
Erman, J. E., & Yonetani, T. (1975b) *Biochim. Biophys. Acta 393*, 343.
Erman, J. E., Vitello, L. B., Mauro, J. M., & Kraut, J. (1989) *Biochemistry 28*, 7992.
Ferrin, T. E., Huang, C. C., Jarvis, L. E., & Langridge, R. (1988) *J. Mol. Graphics 6*, 13.
Finzel, B. C., Poulos, T. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 13027.
Fishel, L. A., Villafranca, J. E., Mauro, J. M., & Kraut, J. (1987) *Biochemistry 26*, 351.
Fishel, L. A., Farnum, M. F., Mauro, J. M., Miller, M. A., & Kraut, J. (1991) *Biochemistry 30*, 1986.
Fox, T., Ferreira-Rajabi, L., Hill, B. C., & English, A. M. (1993) *Biochemistry 32*, 6938.
Goodin, D. B., & McRee, D. E. (1993) *Biochemistry 32*, 3313.
Goodin, D. B., Mauk, A. G., & Smith, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1295.
Goodin, D. B., Mauk, A. G., & Smith, M. (1987) *J. Biol. Chem. 262*, 7719.
Hoffman, B. M., Roberts, J. E., Brown, T. G., Kang, C. H., & Margoliash, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6132.
Hori, H., & Yonetani, T. (1985) *J. Biol. Chem. 260*, 349.
Houseman, A. L. P., Doan, P. E., Goodin, D. B., & Hoffman, B. M. (1993) *Biochemistry 32*, 4430.
Jordi, H. C., & Erman, J. E. (1974) *Biochemistry 13*, 3741.
Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, Plenum, New York.

Mauro, J. M., Fishel, L. A., Hazzard, J. T., Meyer, T. E., Tollin, G., Cusanovich, M. A., & Kraut, J. (1988) *Biochemistry 27*, 6243.
Neumann, N. P. (1967) *Methods Enzymol. 11*, 485.
Nielsen, T. B., & Reynolds, J. A. (1978) *Methods Enzymol. 48*, 3.
Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115.
Prince, R. C., & George, G. N. (1990) *Trends Biochem. Sci. 15*, 170.
Saito, Y., Tachibana, H., Hayashi, H., & Wada, A. (1981) *Photochem. Photobiol. 33*, 289.
Scholes, C. P.; Liu, Y., Fishel, L. A., Farnum, M. F., Mauro, J. M., & Kraut, J. (1989) *Isr. J. Chem. 29*, 85.
Sivaraja, M., Goodin, D. B., Smith, M., & Hoffman, B. M. (1989) *Science 245*, 738.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190.
Spangler, B. D. (1984) Ph.D. Dissertation, Northern Illinois University, Dekalb, IL.
Spangler, B. D., & Erman, J. E. (1986) *Biochim. Biophys. Acta 872*, 155.
Yonetani, T., & Ray, G. S. (1965) *J. Biol. Chem. 240*, 4503.
Yonetani, T., & Anni, H. (1987) *J. Biol. Chem. 262*, 9547.