Cytochrome *c* Peroxidase Compound ES Is Identical with Horseradish Peroxidase Compound I in Iron–Ligand Distances†

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ABSTRACT: X-ray absorption studies of compound ES of cytochrome *c* peroxidase show a short iron–oxygen distance of 1.67 ± 0.04 Å, an iron–histidine distance of 1.91 ± 0.03 Å, and an iron–pyrrole nitrogen average distance of 2.02 ± 0.02 Å. This is identical within the error with the reported structure of horseradish peroxidase compound I [Chance, B., Powers, L., Ching, Y., Poulos, T., Yamazaki, I., & Paul, K. G. (1984) *Arch. Biochem. Biophys. 235*, 596–611]. Comparisons of the structures of myoglobin peroxide [Chance, M., Powers, L., Kumar, C., & Chance, B. (1986) *Biochemistry* (preceding paper in this issue)], compound ES, and the intermediates of horseradish peroxidase reveal the possible mechanisms for the stabilization of the free radical species generated during catalysis. The proximal histidine regulates the structure and function of the pyrrole nitrogens and the heme, allowing for the formation and maintenance of the characteristic intermediates.

Cytochrome *c* peroxidase (ccp)[1] has become a standard in the peroxidase field for the thoroughness of the spectral, kinetic, and structural analyses performed on the native protein and its complex with H_2O_2, called compound ES. Early on, the spectral and kinetic similarities and differences between ccp and horseradish peroxidase (hrp) had been recognized. Reductive titration of ES showed it to retain two oxidizing equivalents per heme (Yonetani, 1965), like hrp compound I. Unlike hrp compound I, ES was red and exhibited an EPR signal typical of a free radical (Yonetani et al., 1966b). Compound ES was proposed to be a Fe^{4+} species and an amino acid free radical (Yonetani et al., 1966b; Yonetani, 1970). Recently, the X-ray crystal structure of ccp at 1.7 Å has emerged (Finzel et al., 1984; Poulos & Finzel, 1984). This has pointed out the environment of the heme in this enzyme along with the amino acids situated in the active site. However, these various techniques have so far failed to provide direct evidence for the complete structure around the iron atom in ccp ES.

We report the X-ray absorption spectrum of compound ES of ccp finding one oxygen atom bonded closely to iron in the sixth position of the heme. The iron–pyrrole nitrogen distances ($Fe-N_p$) and the iron–histidine distance ($Fe-N_e$) resemble those of the native structure. The iron oxidation state is above Fe^{3+} as indicated by X-ray edge measurements. In addition, the structure of ES around the iron is identical, within the error, with that observed for hrp compound I (Chance et al., 1984).

When the structure of ES reported here is compared to that of myoglobin peroxide (Chance et al., 1986) and hrp compounds I and II (Chance et al., 1984), the structural similarities and differences support the general mechanism for peroxidase chemistry proposed earlier (Chance et al., 1986):

$$X\text{–}E\text{–}Fe^{3+} + HOOH \xrightarrow{k_1} X^{+}\text{—}E\text{—}Fe^{4+}{=}O + H_2O \xrightarrow[e^-]{k_3}$$

$$X\text{–}E\text{–}Fe^{4+}\text{–}O \xrightarrow[e^-]{k_5} X\text{–}E\text{–}Fe^{3+} + H_2O$$

where X^+ represents the hrp porphyrin radical, the ccp ES amino acid radical, or an oxidized protein (for myoglobin).

In ccp, like in hrp, the formation of the primary complex, an oxyferryl ion (George & Irvine, 1952), occurs with one electron coming from iron making a Fe^{4+} state, while the second electron equivalent comes from a protein-centered radical in ccp and a π-cation radical in hrp. The oxidized protein complexes are reduced in two single-electron transfer back to native enzyme. The one electron reduced form of ES has not been well characterized (Coulson et al., 1971; Poulos & Finzel, 1984), but the similarities in structure between ES and hrp compound I, despite the difference in radical location, reinforce the importance of the short $Fe-N_e$ bond in the peroxidase compounds in determining the enzyme intermediates. This general feature of the peroxidases contrasts with myoglobin and its peroxide compound, where the longer $Fe-N_e$ bond reduces the transient stability of the π-cation radical (Chance et al., 1986).

Experimental Procedures.

Cytochrome *c* peroxidase was purified according to Nelson et al. (1977) and recrystallized twice by dialysis against distilled water (Yonetani et al., 1966a). Conversion of ccp to ES did not proceed to completion at protein concentrations required for EXAFS studies. Therefore, conversion to ES was carried out in dilute solutions followed by a concentration step.

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[1] Abbreviations: *c*, speed of light; ccp, cytochrome *c* peroxidase; eV, electronvolts; *E*, X-ray energy; E_0, iron edge energy or threshold; EXAFS, extended X-ray absorption fine structure; GeV, gigaelectronvolts; hrp, horseradish peroxidase; keV, kiloelectronvolts; LFIR, ligand field indicator region; N_p, pyrrole nitrogen; N_e, proximal histidine nitrogen.

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Table I

	model	N	r	$\Delta\sigma^2$	ΔE_0	R^2
A	Fe–N	5	1.99	1.1×10^{-3}	−2.0	3.2
	Fe–N	1	1.66	5.5×10^{-3}	−1.5	
B	Fe–N	4	2.02	2.1×10^{-3}	−3.5	15
	Fe–N	2	1.70	5.2×10^{-3}	−8.0	
C	Fe–N	4	2.02	2.4×10^{-3}	1.3	2.0
	Fe–N	1	1.91	6.9×10^{-3}	7.6	
	Fe–O	1	1.66	5.0×10^{-3}	−0.8	
D	Fe–N	4	2.02	-3.3×10^{-3}	−1.4	9.0
	Fe–N	1	2.02	6.6×10^{-3}	−4.2	
	Fe–O	1	1.66	5.8×10^{-3}	−6.2	
E	Fe–N	4	2.02	1.9×10^{-3}	−2.4	15
	Fe–N	1	2.10	*−3.4*	*>100*	
	Fe–O	1	1.66	5.8×10^{-3}	−6.6	
F	Fe–N	4	2.02	1.3×10^{-3}	−1.8	7.4
	Fe–N	1	1.66	-3.1×10^{-3}	*−31.4*	
	Fe–O	1	1.66	-6.3×10^{-3}	−3.4	
G	Fe–N	4	2.02	3.2×10^{-3}	3.5	6.6
	Fe–N	1	1.66	7.3×10^{-3}	2.9	
	Fe–O	1	1.91	2.0×10^{-3}	−2.0	

A 0.36 mM solution of peroxidase in 28% ethylene glycol and 0.1 mM potassium phosphate buffer, pH 6.0, was treated with a 2-fold molar excess of H_2O_2 at 4 °C. The sample was concentrated to 3 mM in a small Amicon ultrafiltration device at 4 °C, requiring a total of 5 h. A few microliters of concentrated ES was diluted into peroxide-free buffer and the absorption spectrum recorded. The characteristic α and β bands were present with the expected Soret maximum of 419 nm (Yonetani et al., 1965), indicating that conversion to ES had proceeded to better than 80% completion. The concentrated sample was immediately frozen on dry ice where ES is indefinitely stable. Reflectance spectrophotometry allowed the recording of optical changes in situ immediately before and after X-ray exposure (Powers et al., 1981). X-ray absorption data for EXAFS and edge studies were collected as described previously. Sample exposure times were 5 h or less. Periodic spectrophotometric monitoring assured the integrity of the preparation.

The data were averaged and analyzed by previously reported procedures (Lee et al., 1981; Chance et al., 1984) and plotted as a function of k as shown in Figure 1. The different ligand shells can be isolated by a Fourier transform of Figure 1, as shown in Figure 2. With a 1.4-Å full-width half-maximum filter, back-transformed data from k = 3.5–12 Å^{-1} contain over seven independent degrees of freedom (Lee et al., 1981). A 20% contribution of native ccp (back-transformed first shell filtered data) was subtracted from the data set to correct for any unreacted material in the sample. No changes within the reported error were seen when the corrected and uncorrected data sets were compared. Figures 1–3 are uncorrected.

A two atom type fitting procedure (Lee et al., 1981) was used to fit the filtered data with FeTPP as a model for Fe–N contributions and iron(3+) acetylacetonate for Fe–O contributions (Powers et al., 1981; Woolery et al., 1985). This procedure represents each atom type by N scatterers at average distance r having a change in Debye–Waller factor $\Delta\sigma^2$ and threshold ΔE_0 from the model. The N values were constrained to their known ratios to reveal possible axial distance solutions (5/1 ratios) and possible Fe–N_p distance solutions (4/2 ratios). This fitting procedure, then, has six unknowns. Parameter space was searched for chemically and mathematically reasonable solutions. The results for ccp ES are shown in Table I. Only one axial and one Fe–N_p solution are found to be reasonable. If the second axial distance is between the two reported solutions, the two-atom procedure may not be able to resolve it as a distinct contribution. The fitting program

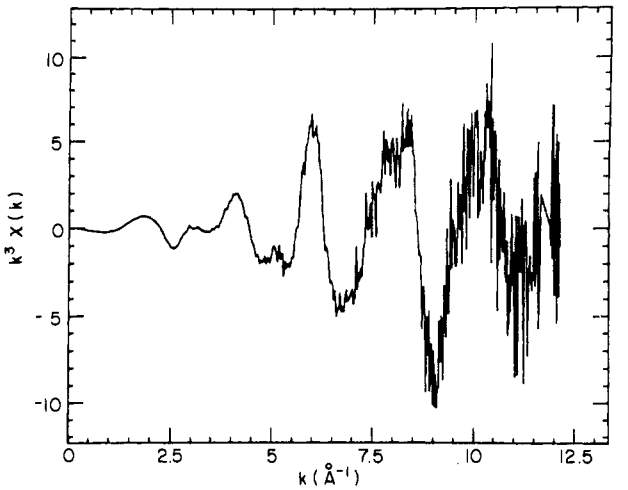

FIGURE 1: EXAFS of ccp compound ES. Fluorescence amplitude normalized to one absorbing atom and multiplied by k^3 vs. k.

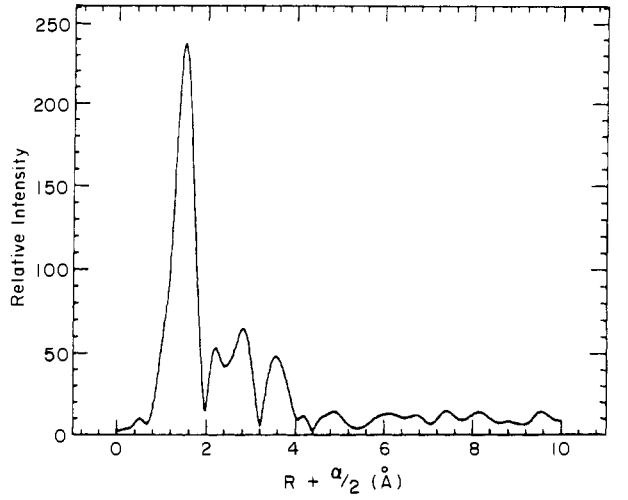

FIGURE 2: Fourier transform of ccp compound ES EXAFS data shown in Figure 1.

will have special problems if the second axial distance is close to Fe–N_p. A three atom type consistency test is then used to establish what approximate solutions taken together were contained in the data. In this test, the N and r values are held fixed, leaving only six unknowns. The results of this consistency analysis are shown in Table I. The errors were determined by changing each r value independently until the R^2

Table II: Structures and Properties of Heme Peroxidatic Compounds

	hrp compd I[a]	ccp compd ES[b]	hrp compd II[a]	myoglobin peroxide[c]	native ccp,[a] hrp,[a] met-MB[c]
Fe–O distance	short	short	long	short	very long, ccp; very long, hrp; long, Met-Mb
Fe–Np distance	expanded	expanded	contracted	contracted	expanded
proximal histidine distance	short	short	long	long	short, ccp and hrp; long, Met–Mb
radical	π-cation	amino acid	none	none	none
color	green	red	red	red	brown
LFIR ratio	1.00 ± 0.03	1.00 ± 0.03	0.84 ± 0.03	0.90 ± 0.03	1.35 ± 0.03
theoretical magnetic moment	3.87,[d] $S = {}^3/_2$	4.56,[d] $S = 1 + S = {}^1/_2$	2.87,[d] $S = 1$	2.87,[d] $S = 1$	5.91,[d] $S = {}^5/_2$
reported magnetic moment	4.00,[a] 4.40[d]	4.10[d]	3.53[a]	2.90[e]	5.5-5.9[a,c]

[a] Chance et al., 1984. [b] This paper. [c] Chance et al., 1986. [d] Yonetani et al., 1970. [e] George & Irvine, 1953.

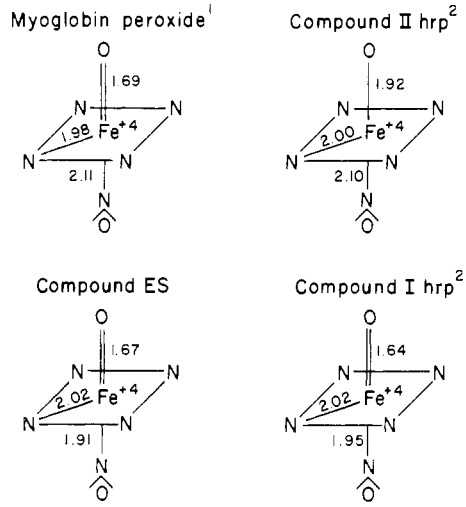

FIGURE 3: Structures of oxidized hydroperoxidase intermediates: (1) Chance et al. (1986); (2) Chance et al. (1984). All Fe–N_p distances are ±0.01–0.02 Å. All other distances are ±0.02–0.03 Å.

increased 2-fold. Complete descriptions of these procedures including comparisons to crystallographic results are given by Powers et al. (1984), Chance et al. (1984), Woolery et al. (1985), and Chance et al. (1986). In all cases studied, the results reported with the three-atom consistency test were equal within the error to the crystallographic results even in the case of small molecules, where the reported crystallographic error is small.

The second shell was analyzed by the two atom type procedure described previously where the partially resolved second and third shells of the heme and proximal histidine contributions are represented as a single contribution (Powers et al., 1981). First, the filtered higher shell data of compound ES were fit with that of native ccp having amplitudes constrained to represent the heme and proximal histidine carbon contributions. Second, an additional oxygen atom [iron(3+) acetylacetonate] was then introduced by the two-atom fit with the amplitude constrained and the sum of residuals compared to the fit without the additional atom. If the R^2 decreased by a factor of 10 or more, then the additional contribution was judged necessary.

Results

The edge spectrum of compound ES was compared with the edge of native ccp reported previously (Chance et al., 1984). An edge shift of 1.2 ± 0.5 eV to a higher energy was observed for compound ES compared to native ccp. This indicates that the iron atom in ES is oxidized by up to one electron above Fe^{3+} by comparison with the shifts observed in hrp (Chance et al., 1984), myoglobin peroxide (Chance et al., 1986), and oxymyoglobin (L. Garcia-Iniquez, L. Powers, and B. Chance, unpublished observations).

The LFIR for ES and other heme compounds is shown in Table II. The LFIR ratio is reduced considerably in the oxidized heme peroxide compounds compared to the native high-spin ferric precursors. These spectral features arise from interference of second and third shell ligands (M. Chance, unpublished observations) but do correlate with the known spin state of the compound (Chance et al., 1984). As such, it provides a spectral assignment of the compound studied and an internal control in each scan for the authenticity of the species in addition to optical verification.

The comparisons of the filtered first shell data with model compounds gave the solutions shown in Table I. The results are reasonable for an iron–oxygen double bond in A. Both two scatterer solutions suggest an Fe–N_p distance that is contracted slightly from the value found for native ccp (Chance et al., 1984). The consistency test confirmed the combination of solutions shown in C. Other combinations using the reported two scatterer distances are shown to be mathematically or chemically unreasonable (Chance et al., 1986; Woolery et al., 1985) in Table I (italic values). The only Fe–N_e value that was consistent with the two-atom solutions was 1.91. Longer or shorter distances for Fe–N_e were not consistent with the two-atom results (solutions D–F). In addition, when the Fe–N_e value was allowed to float (holding all N and the other two r values fixed), it found a minimum at 1.91 in each case. Iteration of solution C by allowing each r value to vary individually assured a minimum if the distances changed by no more than ±0.03 Å. Solution G tests the sensitivity of the procedure to the switching of the O and N distances. To be consistent with the results of the two-atom procedure, the R^2 for solution G must be less than or equal to that of solution A. In addition, solution G is not a minimum. Upon iteration, it moves the r values far from their two-atom values. In summary, solution G is not as good a solution to the data as solution C, which maintained values of r close to the two-atom distances upon iteration. The results are four nitrogens at an average distance of 2.02 ± 0.02 Å, one nitrogen at 1.91 ± 0.04 Å, and one oxygen atom at 1.67 ± 0.02 Å. Solution C is indistinguishable from results for hrp compound I within our fitting error but quite different from those for hrp compound II and myoglobin peroxide (see Figure 3).

The higher shell analysis of compound ES shows that there is no indication of an oxygen atom in the second shell. When the higher shell of native ccp is fit to the higher shell of ES, the addition of an oxygen atom to the simulation worsens the fit. In other words, if ES was an iron peroxide, instead of the oxyferryl species, the addition of an oxygen in this analysis should improve the fit 10-fold (Powers et al., 1984).

Discussion

The peroxidase mechanism demands that two electrons are donated to H_2O_2 in the formation of the first stable complex. In myoglobin, the second equivalent comes from irreversible

oxidation of the protein (Kelso et al., 1963) with no observed stable radical species. In hrp and ccp, nature has designed the protein structures in such a way that the reaction with peroxide is fast, the second equivalent is provided by a reversible stable radical state, and the protein is not irreversibly oxidized during the reaction. One equivalent is provided in all known cases by the iron atom, which is oxidized to a stable Fe^{4+} state. Figure 3 shows the structures of four oxyferryl ions that have been studied by EXAFS (Chance et al., 1984, 1986). A comparison of the optical, magnetic, and kinetic data reported for these compounds reveals the possible relationship of structure to these physical properties.

The Fe^{4+}, $S = 1$ assignment for ES is supported by Mössbauer studies (Oosterhuis & Lang, 1973), our edge studies, and magnetic susceptibility measurements (Theorell et al., 1952; Yonetani, 1970). In Table II, magnetic and structural properties of native and oxidized hydroperoxidases are compared. The magnetic moments of these compounds vary depending on the presence and nature of the radical species. ES has an uncoupled radical, and the iron $S = 1$ should add to the moment of the $S = {}^1/_2$ radical, producing a theoretical moment of 4.56 μ_B. The reported value is slightly lower. Myoglobin peroxide, which has been shown by Mössbauer (Maeda et al., 1971), NMR (La Mar et al., 1983), resonance Raman (Sitter et al., 1985), and X-ray edge measurements (Chance et al., 1984) to be a Fe^{4+} species with no coupled radical, has the expected magnetic moment of 2.8 μ_B (George & Irvine, 1953). Hrp compound I also has a Fe^{4+} state. Mössbauer measurements have shown that the $S = 1$ iron is coupled to a $S = {}^1/_2$ radical (Schulz et al., 1979), although weakly, so that the reported magnetic moment values are slightly above the theoretical magnetic moment of 3.87 μ_B (see Table II). X-ray absorption experiments (Chance et al., 1984) confirm the Fe^{4+} state. Hrp component II's iron oxidation state is also certainly Fe^{4+} as revealed by X-ray absorption (Chance et al., 1984), Mössbauer (Schulz et al., 1984), and resonance Raman experiments (Rakshit & Spiro, 1976). Its observed magnetic moment is higher than the theoretical value of 2.9 μ_B, but the error in the magnetic moment measurements can be large. All these compounds are variants of the oxyferryl species first proposed by George (George & Irvine, 1953).

The green color of compound I (Theorell, 1941) is due to its porphyrin radical (Dolphin et al., 1972). Compound ES, in color, resembles compound II of hrp and myoglobin peroxide, which also lack porphyrin radicals. The identical structures reported here for compound I and ES strongly supports this assignment of the green color to the porphyrin radical, as the only difference between the two structures is the radical location.

The structures of these four oxyferryl ions are shown in Figure 3. The similarities between ES and hrp compound I are clear, and the differences between these and the other two compounds, which lack radicals, are large. Compound II of hrp and myoglobin peroxide both have shortened $Fe-N_p$ and longer $Fe-N_e$ distances. Even though ES has a different radical, it is unaltered in structure from compound I. Fast optical experiments (Chance et al., 1969) have given evidence for the existence of a green intermediate in the reaction of H_2O_2 and ccp preceding the formation of the red ES. It is likely that this green species is a transient porphyrin radical, which then transfers quickly to the amino acid radical (Fujita et al., 1983). This hypothesis may help explain why the structures of hrp compound I and ES are so similar, since in the initial stage of its formation ES is presumed to be identical with hrp compound I. The question remains, why does the structure of ES not change when the radical shifts its location?

In hrp, we have proposed (Chance et al., 1986) that, when compound I is reduced to compound II, the change in A_{2u} molecular orbital occupancy (Rutter et al., 1983) is reflected in a slight contraction of the $Fe-N_p$ distance and a lengthening of the axial orbitals. This structure is shown in an extreme way in myoglobin peroxide, where the $Fe-N_p$ distance is very short. This change to a more contracted pyrrole nitrogen distance is not seen in ES, which also has a filled in porphyrin radical. Possibly, there is significant overlap between the amino acid radical orbitals, the histidine, and the pyrrole nitrogens, providing stabilization for the radical. Such a delocalization may be unlikely, considering that the EPR signal indicates the ES radical has a spin concentration equal to that of the heme (Yonetani et al., 1966b). Alternatively, the green ccp intermediate may not as closely resemble hrp compound I as ES does, and when the radical moves, the structure relaxes to one identical with that of hrp compound I. The structure of the green intermediate is not directly known and may be crucial to understanding the logic of the structure of ES.

The short histidine bond in both native ccp and hrp (Chance et al., 1984) directs electron density into the heme in the formation of the primary complexes, making $Fe-N_p$ relatively large. The alternative case, the formation of myoglobin peroxide, occurs with a long histidine that cannot direct electron density into the heme to stabilize any porphyrin radical that might have formed (Chance et al., 1986). Unfortunately, the kinetics of the reaction with myoglobin ($k_1 \ll k_3$) makes the observation of such a transient radical state difficult (B. Chance, unpublished observations).

Poulos and Finzel (1984) have provided a rationale for the greater stability of an amino acid radical in ccp, compared to a porphyrin radical, as in hrp. When ES is reduced, it does not preferentially fill in the iron of the radical and there is a fast equilibration between sites for the one electron reduced form (Coulson et al., 1971). In hrp, the first electron goes to the porphyrin radical, and there is no equilibration between sites. These differences are more readily explained by the extra aromatic stabilization afforded by the ccp pocket relative to hrp than by the minute structural differences around the iron in ES and hrp compound I. Residues Trp-51, Trp-191, and Met-172 are strategically located to provide an environment richer in π-orbitals than that of hrp. The presence of extra stabilizing groups around the site of the ccp radical makes the stability of the iron and the radical sites about equal, allowing for the fast equilibration or delocalization (Poulos & Finzel, 1984). In hrp, the porphyrin site is less stable than the iron site, and it is filled in first and stays filled. A similar situation exists for myoglobin peroxide, since the one electron equivalent resides on the iron, not in a porphyrin radical, or delocalized between them.

That the histidine may play an important role in regulating the structure and function of the pyrrole nitrogens and the heme, in order to make possible the specific chemistry of the peroxidases, is suggested by these results. The different heme pocket in ccp also provides the environment for stabilization of the compound ES radical in the one electron reduced form. This kinetic difference is certainly of physiological importance and deserves further study.

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Registry No. Ccp, 9029-53-2; hrp, 9003-99-0; Fe, 7439-89-6; N_2, 7727-37-9; O_2, 7782-44-7.

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