Magnetic Circular Dichroism Studies on the Heme and Tryptophan Components of Cytochrome c Peroxidase*

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David Myers and Graham Palmer
From the Department of Biochemistry, Rice University, Houston, Texas 77251

We have measured the magnetic circular dichroism of cytochrome c peroxidase and some of its derivatives from 250–350 nm. Comparison of the changes observed on conversion to the catalytic intermediate (cytochrome c peroxidase-I) with spectra obtained from horseradish peroxidase and its derivatives and model compounds of protoporphyrin leads us to the conclusion that the observed changes in the magnetic circular dichroism spectra reflect conversion of the heme to the ferryl state. No evidence was found for modification of tryptophan in cytochrome c peroxidase-I.

Cytochrome c peroxidase is a heme containing enzyme found in bacteria and yeast (1). The yeast enzyme reacts with hydrogen peroxide to form an intermediate, cytochrome c peroxidase-I, which is two oxidizing equivalents above the resting enzyme (2, 3). One of the oxidizing equivalents is carried by the heme iron, which is present at the ferryl oxidation level. The second oxidizing equivalent is believed to be present on an amino acid side chain.

The amino acid that bears the free radical is undetermined (2–9). Both x-ray crystallographic modelling (4–6) and analysis of optical spectra (3) have been interpreted in terms of an indole radical present on a tryptophan residue, while Hoffman (7–9) has argued that two amino acids, one a methionine, are involved.

MCD in the UV region can be used to quantitatively measure tryptophan content in proteins. It is a rapid, nondestructive, and apparently free of interference from other aromatic amino acids (10). Consequently, we have examined the ultraviolet MCD behavior of cytochrome c peroxidase with the objective of establishing whether tryptophan is the source of the free radical present in cytochrome c peroxidase compound I. To account for potential contribution from heme absorption in this region, derivatives and apoproteins of both cytochrome c peroxidase and horseradish peroxidase were prepared, along with heme model compounds, and their UV MCD spectra were characterized.

MATERIALS AND METHODS

Cytochrome c peroxidase was prepared according to the method of Nelson and was crystallized at least once (11). The ratio Am/Am of several preparations ranged from 1.25 to 1.35 and the activity of the enzyme (mol of cytochrome c oxidized/mol of cytochrome c peroxi-
dase) varied from 900 to 1200 s\(^{-1}\) when assayed in 0.05 M sodium acetate, pH 6.0, at 25 °C, using 20 μM horse heart cytochrome c (Sigma type VI) and 170 μM H\(_2\)O\(_2\) as substrates. The absorption coefficient of the preparations at 408 nm was 98 m\(^{-1}\) cm\(^{-1}\), based on pyridine hemochromogen analysis of the heme content. Inclusion of 0.7 mm phenylmethylsulfonyl fluoride in the original autolysis suspension reduces the low-spin heme content to less than 5% as judged by EPR (9). Otherwise, substantial amounts of low-spin heme were found to be present in the enzyme as isolated.

Cytochrome c peroxidase-I was prepared by adding a 10% molar excess of hydrogen peroxide. Optical and MCD spectra were taken immediately. Then, a 75–150 molar excess of dithiothreitol was added to the compound I to regenerate the ferric enzyme. MCD and optical spectra were then again recorded.

Horse-radish peroxidase was purchased from Boehringer Mannheim and purified according to the method of Shannon et al. (12). The purified peroxidase had a Am/Am ratio of 3.3. Horseradish peroxidase compound II was prepared according to the method of Stillman et al. (13). Optical and MCD measurements were taken of a horseradish peroxidase sample in 0.05 M borate buffer, pH 9.0, which was then treated with an equimolar amount of H\(_2\)O\(_2\) and allowed to stand for at least 24 h to destroy reducing equivalents present in the solution. The treated peroxidase was subsequently exposed to 1.2 eq of H\(_2\)O\(_2\) and then to 0.8–1.0 eq of potassium ferrocyanide. MCD spectra of the resulting compound II were recorded immediately. Optical spectra recorded before and after MCD measurements show a loss of 5% absorption in the Soret of compound II during the course of the MCD measurement. Spectra of carefully measured aliquots of potassium ferrocyanide were subtracted from the horseradish peroxidase-II MCD spectra to compensate for MCD absorption of ferri cyanide produced during horseradish peroxidase-II formation.

Apoproteins of both cytochrome c peroxidase and horseradish peroxidase were prepared by the method of Yonetani (14). The UV MCD was recorded, and the molar concentrations of the apoproteins were determined using the known tryptophan content (15–19). The MCD were compared with MCD of native enzyme and their cyanide and fluoride derivatives and difference spectra were calculated.

Hemin chloride was purchased from Sigma and dissolved in dimethyl sulfoxide. Imidazolin-1-ylmethylidazole, 2-methylimidazolone, or 1,2-dimethylimidazolone were added to this stock heme solution to a final concentration of 0.1 M. The UV MCD spectra of the resultant solutions was recorded in 0.1-cm path length cells to minimize the absorbance of the dimethyl sulfoxide. Concentrations of heme were determined by pyridine hemochromogen assay (20). Optical spectra were recorded on a Cary Model 17, and MCD spectra were recorded on a Jasco J500C equipped with a 1.3 tesla electromagnet. Both instruments were linked to a minicomputer for data processing and further manipulation. The MCD spectrometer was calibrated in CD and MCD modes with standardized solutions of camphor/sulfonic acid and potassium ferrocyanide, respectively, N-acetyl tryptophanamide was used as a tryptophan standard (10). EPR spectra were recorded on a Varian E9 spectrometer as before (21).

RESULTS

The ultraviolet MCD spectrum of native cytochrome c peroxidase is typical of tryptophan containing enzymes, and the intensity at 290 nm accounts for 7.5 ± 0.6 mol of trypto-
phane/mol of enzyme (average of 5 preparations). This should be compared with a value of 7 obtained from sequence analysis (17, 18). The addition of 1.1 eq of hydrogen peroxide converts cytochrome c peroxidase to cytochrome c peroxidase-I and causes partially reversible changes in both the Soret and UV regions (Fig. 1). The g = 6 EPR signal due to the high-spin ferric heme is reduced to about 1% of its original amplitude, and an intense EPR, characteristic of cytochrome c peroxidase-I, appears at g = 2. In the UV, the MCD intensity decreases by an amount equivalent to about 1.5 tryptophan.

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1 The abbreviations used are: MCD, magnetic circular dichroism; MES, 4-morpholinolinesulfonic acid.
Addition of dithiothreitol to cytochrome c peroxidase-I reduces the enzyme back to the ferric state, and restores about 65% of the original loss of MCD absorption. This is in contrast to experiments in which cytochrome c peroxidase-I was incubated for prolonged periods (80 h to 2 weeks) in the absence of any exogenous reductant. It has been previously reported that such incubations return cytochrome c peroxidase to a "native like state," with 70% of its original activity (22-24). Although an apparently native optical spectrum is obtained in the Soret region after a prolonged incubation, the decrease in UV MCD intensity is not completely restored. EPR spectra show that the $g = 2$ signal of cytochrome c peroxidase-I has been eliminated. However, only a proportion of the $g = 6$ high-spin signal is recovered. In addition, a new low-spin signal ($g_1 = 1.78$, $g_2 = 2.29$, $g_3 = 2.64$) is observed. This new low-spin signal is estimated to account for as much as one-third of the heme content. As low-spin hemes appear to have much more negative MCD than high-spin hemes (see below), the partial conversion of the heme center of cytochrome c peroxidase from high spin to low spin provides a satisfactory explanation for the incomplete recovery of MCD intensity as cytochrome c peroxidase-I is converted back to cytochrome c peroxidase.

Conversion of HRP to HRP-II also causes a decrease of MCD intensity in the UV (see below). Since the fundamental difference between native horseradish peroxidase and horseradish peroxidase compound II is believed to be the oxidation state of the iron, and no role has previously been suggested for tryptophan in this enzyme, this loss of MCD absorption

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3 Because heme absorbs in the 290 nm region, the possible contribution due to the oxidation of iron (III) to iron (IV) 11, 12.

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**Figure 1.** Optical and MCD spectra of cytochrome c peroxidase (CCP), CCP-I, and rereduced CCP at 15 °C. 7.9 μM CCP was reacted with 8.5 μM H₂O₂ in potassium phosphate buffer, pH 6.0. Subsequently, 1 mM dithiothreitol was added.

**Figure 2.** MCD spectra of horseradish peroxidase (HRP) and HRP-II at 15 °C. See "Materials and Methods" for details on the preparation of HRP-II.

However, the shape of the cytochrome c peroxidase minus cytochrome c peroxidase-I difference spectrum does not resemble the absolute spectrum of N-acetyltryptophanamide (Fig. 3), suggesting that the observed loss of intensity is not due to the elimination of a proportion of the tryptophan contribution.
UV MCD of Cytochrome c Peroxidase

Fig. 3. MCD difference spectra of cytochrome c peroxidase (CCP) minus CCP-1 (left) and horseradish peroxidase (HRP) minus HRP-II (center) compared to the absolute spectrum of N-acetyltrypophanamide (right). The difference spectra were calculated from absolute spectra obtained in experiments similar to those described in Figs. 1 and 2. The spectrum of N-acetyltrypophanamide was obtained in 0.05 M MES, pH 6.0, at 20 °C.

Fig. 4. The absolute MCD spectra of the fluoride and cyanide complexes of cytochrome c peroxidase (CCP) and horseradish peroxidase (HRP). 8.0 μM CCP was incubated with either 0.25 M KF or 20 mM NaCN; 20 μM HRP was incubated with 0.25 M KF 0.1 M NaCN. The buffer was 0.05 M MES in all cases and the temperature 20 °C.

can be reasonably attributed solely to the oxidation of ferric to ferryl heme.

The difference spectrum of cytochrome c peroxidase minus cytochrome c peroxidase-I (Fig. 3, left) bears a clear similarity to the difference spectrum of horseradish peroxidase minus horseradish peroxidase-II (Fig. 3, center). MCD spectral changes caused by transformation of ferric cytochrome c peroxidase to a radical-containing, ferryl cytochrome c peroxidase-I appear to be duplicated by the ferric to ferryl transformation of horseradish peroxidase to horseradish peroxidase-II, horseradish peroxidase-II being a species without any radical. The similarity of the changes in the cytochrome c peroxidase-I and horseradish peroxidase-II difference spectra suggests a common cause for the perturbations. Thus the changes observed in the formation of the cytochrome c peroxidase-I MCD absorption appear to be accounted for satisfactorily by conversion of heme to the ferryl state, and no evidence for formation of an indole radical can be drawn from these MCD experiments. Data from cytochrome c peroxidase-I and horseradish peroxidase-II suggest heme spin/oxidation
state changes are responsible for the UV effects. Accordingly, several model systems were examined to investigate this possibility.

MCD spectra of cytochrome c peroxidase, horseradish peroxidase, their cyanide and fluoride derivatives, and their respective apoproteins were recorded from 350–250 nm (Fig. 4). The contributions of the heme centers were extracted by subtracting the data for the apoprotein from the other samples. The cytochrome c peroxidase and horseradish peroxidase difference spectra so obtained are quite similar (Fig. 5). The difference spectrum of cytochrome c peroxidase-CN shows a bilobal negative MCD spectrum, whereas the high-spin bis-dimethyl sulfoxide derivative displays a non-bilobal negative MCD spectrum, while difference spectra of both cytochrome c peroxidase and cytochrome c peroxidase-F exhibit positive MCD. Since native cytochrome c peroxidase and horseradish peroxidase are predominantly high spin, while the cyanide derivatives are low spin (1, 15), the sign of ferric heme contributions is apparently dependent on spin state.

MCD spectra of heme model compounds support this conclusion (Fig. 5). The spectra of six-coordinate, protoheme-ligand complexes in dimethyl sulfoxide show features remarkably similar to the enzyme minus apoprotein spectra. Thus low-spin bis-imidazole protoheme has a negative bilobal MCD spectrum, whereas the high-spin bis-dimethyl protosulfoxide heme solution exhibits positive MCD with a maximum around 320 nm. The MCD of high-spin protoheme in the UV region also appears to be dependent on the sixth coordinating ligand. For example, the maxima of bis-dimethyl sulfoxide protoheme, 1,2-dimethylimidazole protoheme,3 horseradish peroxidase fluoride, and aquo-horseradish peroxidase are all at significantly different wavelengths, with noticeably different amplitudes, despite all these species being substantially high spin. Similar behavior has been obtained with a variety of high-spin heme a derivatives in the Soret region (21).

In summary, no effects in the MCD spectra can be attributed to radical formation by tryptophan. The data suggest spin-state or oxidation-state changes in the heme as the cause of the spectral variation. Model studies support this conclusion. The observed changes in MCD intensity in the model compounds are much larger than the changes in the cytochrome c peroxidase-I or horseradish peroxidase-II difference spectra and provide a simple rationale for the loss in MCD intensity upon formation of the ferryl-containing catalytic intermediate cytochrome c peroxidase-I.

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3 Protoheme dissolved in a solution containing 0.1 M 1,2-dimethylimidazole is nearly 100% high spin at room temperature (24). Presumably, the complex is five coordinates under these conditions.