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# CIRCULAR DICHROISM OF HORSERADISH PEROXIDASE AND ITS ENZYME–SUBSTRATE COMPOUNDS

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## SUMMARY

The conformations of peroxidase ($HNO_2$:$H_2O_2$ oxidoreductase, EC 1.11.1.7), peroxidase–substrate compound I, and peroxidase–substrate compound II were investigated using circular dichroism. Peroxidase has positive circular dichroic bands at 190 and 406 m$\mu$ and negative bands at 207, 222, 282, 335, and 370 m$\mu$. The far ultraviolet, circular dichroic spectrum of peroxidase resembles that of $\alpha$-helical polypeptides. The intensities of the 207- and 222-m$\mu$ bands are about 40% of the literature values for completely $\alpha$-helical polypeptides. A mixture of the two enzyme–substrate compounds has the same 207- and 222-m$\mu$ circular dichroic bands as does free peroxidase, suggesting that all three forms have the same secondary structure. In the Soret region, the formation of enzyme–substrate compounds shifts the wavelengths of the circular dichroic bands; these changes are similar to those known to occur in the absorption bands. Also in the 280-m$\mu$ region, the circular dichroism of the enzyme–substrate compounds differs from that of peroxidase, indicating either that the heme moiety contributes to the ultraviolet circular dichroism or that the orientation of an aromatic amino acid residue changes during peroxidatic activity.

## INTRODUCTION

Interest in the mechanism of enzyme action has led many investigators to search for conformation changes which might occur in enzymes during catalysis[1–4]. Direct evidence of such conformation changes, however, has been difficult to obtain. Recently advances in circular dichroic (CD) instrumentation[5] have provided a sensitive method to study enzyme conformation during catalysis. The CD technique reveals many aspects of protein structure, such as $\alpha$-helix[6], $\beta$-structure[7–9], and the conformations of certain amino acid residue side chains[10,11].

Abbreviations: CD, circular dichroic; $\Delta A$, absorbance for left circularly polarized light *minus* absorbance for right circularly polarized light; $E$–$S_I$, primary peroxidase–substrate compound; $E$–$S_{II}$, secondary peroxidase–substrate compound.

This paper describes studies on the circular dichroism of horseradish peroxidase ($HNO_2$:$H_2O_2$ oxidoreductase, EC 1.11.1.7) and two of its enzyme–substrate compounds. Horseradish peroxidase was selected because the presence of its enzyme–substrate compounds can be verified by independent measurements of absorption spectra in the visible region[12,13]. Two peroxidase–substrate compounds appear to be intermediates in the peroxidatic reaction[13], described by the following mechanism[14]:

$$\text{Peroxidase–Fe}^{3+}\ (H_2O) + H_2O_2 \xrightarrow[2\text{ equiv ox.}\quad (E\text{–}S_I)]{k_1} \text{peroxidase–FeO}^{3+} + 2\ H_2O$$

$$\text{Peroxidase–FeO}^{3+} + AH_2 \xrightarrow[1\text{ equiv red.}\quad (E\text{–}S_{II})]{k_2} \text{peroxidase–FeO}^{2+} + AH\cdot + H^+$$

$$\text{Peroxidase–FeO}^{2+} + AH_2 + H^+ \xrightarrow[1\text{ equiv red.}]{k_3} \text{peroxidase–Fe}^{3+}\ (H_2O) + AH\cdot$$

where $AH_2$ = hydrogen donor, $AH\cdot$ = free radical formed from the donor, $E$–$S_I$ = primary peroxidase–substrate compound, $E$–$S_{II}$ = secondary peroxidase–substrate compound. During the steady state, $E$–$S_{II}$ is the principal intermediate present, because in the presence of excess peroxide and excess donor the first two steps are rapid compared to the last one.

## EXPERIMENTAL

Circular dichroism was measured with a 1966 model of the JASCO CD spectrophotometer distributed in the U.S.A. by the Durrum Co., Palo Alto, Calif. In the visible and near ultraviolet region, high enzyme concentrations (30–60 $\mu$M) were required for the optimum signal-to-noise ratio. Typical instrument tracings are presented in the figures so that the signal-to-noise ratio can be visualized under all experimental conditions. The experiments were repeated at least 4 times to verify reproducibility of the findings presented.

Horseradish peroxidase was obtained from Sigma Chemical Co. (St. Louis, U.S.A.) as a salt-free powder (Type VI), The ratio of peroxidase absorbance at 403 m$\mu$ to that at 275 m$\mu$ was 3.0. Peroxidase concentrations were determined from the absorbance at 403 m$\mu$ ($\varepsilon_{mM}$ 107.7 (ref. 15)).

The peroxidatic reaction was carried out at pH 7 using $NaNO_2$ as the hydrogen donor[16]. These conditions slowed the reaction enough to permit recording CD spectra at high enzyme concentrations. $NaNO_2$ was selected as the hydrogen donor because it does not have circular dichroism.

The conversion of peroxidase into $E$–$S_I$ and $E$–$S_{II}$ was determined from the absorption spectra in the Soret region[13]. A mixture containing about 50% $E$–$S_I$ and 50% $E$–$S_{II}$ was obtained by adding $H_2O_2$ (50–200 $\mu$M) to peroxidase (30–60 $\mu$M). This state, which persisted for more than 15 min, was used to evaluate the CD spectrum of $E$–$S_I$. It could not be obtained in a pure state because the peroxidase solutions contained enough endogenous hydrogen donor[13] to permit forming some $E$–$S_{II}$. This mixture of $E$–$S_I$ and $E$–$S_{II}$ was converted entirely to $E$–$S_{II}$ by adding $NaNO_2$ (100–200 $\mu$M). The pure $E$–$S_{II}$ could be maintained for as long as 4 min.

RESULTS

*Circular dichroism of peroxidase*

CD spectra in the far ultraviolet provide information about the conformation of the peptide backbone. Peroxidase has negative CD bands at 207 and 222 mμ (Fig. 1) and a positive CD band at 190 mμ (not shown). This CD spectrum resembles that reported for α-helical polypeptides[6] and the α-helical proteins ferrimyoglobin[6] and myosin[17]. It differs from those observed for β-structure [7-9] and random coil[6]. Thus peroxidase may have many helical regions. An estimate of about 40% helix is obtained, if synthetic polypeptides are used as standards for 0 and 100% helix[6].

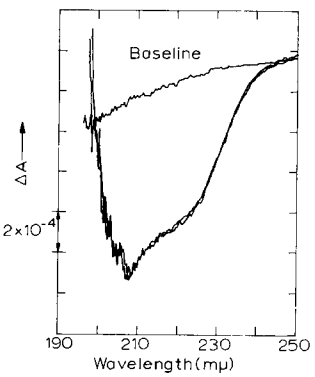


Fig. 1. Record of far ultraviolet CD spectrum of peroxidase and peroxidase–substrate compounds. First trace was peroxidase in 10 mM sodium phosphate, pH 7. Then 50 μM $H_2O_2$ was added to the cuvette and the spectrum was retraced. The two traces are identical within experimental error. Enzyme concentration in both cases was 6.3 μM. Scanning speed: 4.5 mμ/min. Slit: 1.5 mm. Path length: 1.0 mm. Temp. = 25°.

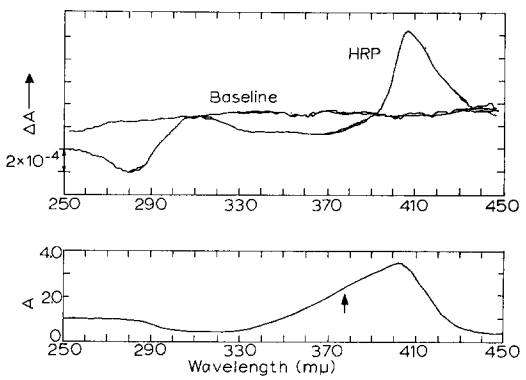


Fig. 2. Record of ultraviolet and visible CD spectrum of horseradish peroxidase (HRP) (top) and drawing of its absorption spectrum (bottom). 32 μM peroxidase in 10 mM sodium phosphate, pH 7. Retrace of CD spectrum shows high instrument stability. Arrow in absorbance diagram indicates the location of a weak absorption band. Scanning speed: 9 mμ/min. Slit: 1.0 mm. Path length: 10.0 mm. Temp. = 25°.

Peroxidase has additional CD bands at longer wavelengths (Fig. 2). Associated with the Soret absorption band is a positive CD band at 406 mμ. Negative CD bands occur at 282, 335, and 370 mμ. The heme group must be responsible for the 335-, 370-, and 406-mμ CD bands because the protein moiety does not absorb in this region[18]. The CD bands at 335 mμ and 370 mμ illustrate the well-known phenomena that weak absorption bands can have appreciable circular dichroism[10,19]. The source of the 282-mμ CD band is considered in DISCUSSION.

*Circular dichroism of enzyme–substrate compounds*

To investigate the possibility of a conformation change occurring during catalysis, the CD bands of the enzyme–substrate compounds were compared with those of free peroxidase. Fig. 1 shows that the far ultraviolet CD spectrum of a mixture of about 50% $E$–$S_I$ and 50% $E$–$S_{II}$ is the same as that of the original enzyme. Unfortunately the relatively high noise level at these wavelengths necessitated scan-

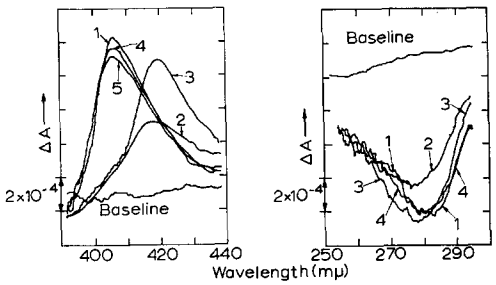


Fig. 3. CD spectra during peroxidatic activity. Separate experiments were made to record spectra (left) in the Soret region (enzyme concentration, 44 $\mu$M) and (right) in the ultraviolet region (enzyme concentration, 67 $\mu$M). No. 1 trace, peroxidase solution in 10 mM sodium phosphate, pH 7; No. 2 trace, 100 $\mu$M $H_2O_2$ was added to the peroxidase used for trace No. 1 and CD spectrum was rescanned immediately; No. 3 trace, 200 $\mu$M $NaNO_2$ was added to solution used for trace No. 2 and CD spectrum was rescanned immediately; No. 4 trace, retrace after completion of reaction; No. 5 trace (in left figure only), retrace after a second cycle of peroxidatic activity. $H_2O_2$ and $NaNO_2$ do not have circular dichroism. Scanning speed: 13 m$\mu$/min. Slit: 1.0 mm Path length: 10.0 mm. Temp. = 25°.

ning the wavelength too slowly to record the far ultraviolet CD spectrum of pure $E$–$S_{II}$ during its transient existence.

More extensive CD measurements were possible at longer wavelengths because the low noise level permitted relatively rapid wavelength scanning. Fig. 3 shows the CD spectra of peroxidase during a cycle of peroxidatic activity. After recording the CD spectrum of peroxidase (trace No. 1), a mixture of $E$–$S_I$ (about 50%) and $E$–$S_{II}$ (about 50%) was formed by adding $H_2O_2$ to peroxidase. This mixture of $E$–$S_I$ and $E$–$S_{II}$ has a weak CD band at 420 m$\mu$ and another band at 278 m$\mu$ (trace No. 2). The latter band is about 20% less intense than the corresponding 282-m$\mu$ CD band in peroxidase. Next pure $E$–$S_{II}$ was formed by adding $NaNO_2$. $E$–$S_{II}$ has a positive CD band at 421 m$\mu$ and a negative CD band at 278 m$\mu$ (trace No. 3). The intensities of these bands are the same as those of the corresponding bands in peroxidase, though the wavelengths are shifted. Finally after the short time needed for the $H_2O_2$ to be reduced, the free enzyme was regenerated, as indicated by the restoration of the original CD spectrum of peroxidase (trace No. 4).

*Absorption changes during peroxidatic activity*

To aid in interpreting the CD spectra, independent spectrophotometric experiments were made to determine the shift in the absorption spectrum occurring during the peroxidatic sequence described above. In the Soret region, the enzymatic activity alters the absorption intensity and shifts the peak in essentially the same way as described for the CD spectrum. In the ultraviolet region, the absorption of the enzyme–substrate compounds differs only slightly from that of peroxidase. For example, at 280 m$\mu$, the mixture of $E$–$S_I$ and $E$–$S_{II}$ has 5% less absorbance than peroxidase; $E$–$S_{II}$ 5% more absorbance than peroxidase.

## DISCUSSION

The conformations of peroxidase and two of its enzyme–substrate compounds

were examined using circular dichroism. The intensity of each CD band is influenced both by the chromophore and by its dissymmetry. Either the chromophore is intrinsically dissymetric or the environment around the chromophore is dissymetric[19]. The CD bands observed in the present investigation result from conformational dissymmetry, since they disappear when peroxidase is denatured (unpublished experiments). By studying the CD bands of various chromophores, several aspects of peroxidase conformation may be revealed.

If the conformation of peroxidase does change during peroxidatic activity, the active site might be the most likely place to observe the altered conformation. For this reason, the heme CD bands will be considered first. The Soret CD band of $E$–$S_{II}$ is the same intensity as that of peroxidase, but the wavelength is shifted to 421 m$\mu$. In contrast, the Soret CD band in the mixture of $E$–$S_I$ and $E$–$S_{II}$ is much less intense than the corresponding band in either $E$–$S_{II}$ or peroxidase. These findings indicate that the Soret CD band of $E$–$S_I$ is less intense than that of either $E$–$S_{II}$ or peroxidase. Unfortunately, this result does not indicate whether the conformation around the heme in $E$–$S_I$ is altered, since its Soret absorption band is also less intense. In these experiments, altered CD bands can be interpreted unambiguously only when the absorption band is unaltered, because the CD intensity is influenced both by the chromophore and the dissymmetry[19]. Since changes in CD intensity during the formation of $E$–$S_I$ are similar to the changes in absorbance for unpolarized light, the new CD spectrum simply may reflect the new electronic transitions involved in absorption. On the other hand, nothing in these data excludes the possibility of a conformation change having occurred around the heme in $E$–$S_I$.

In view of this ambiguity, it is important to consider additional CD bands which may reflect other aspects of protein conformation. Aromatic amino acid residues may be used to probe for alterations in the local conformation of the protein moiety, because their CD bands are far removed from the major heme CD bands discussed above. Since both tyrosine and tryptophan have CD bands near 280 m$\mu$ (ref. 10), the 282-m$\mu$ CD band in peroxidase deserves special consideration. Its intensity may reflect the orientation of either tyrosyl of tryptophanyl moieties. Phenylalanyl and cystyl moieties probably can be ruled out, because their CD bands have not been observed at such long wavelengths (but see ref. 10). If the 282-m$\mu$ CD band is caused by the orientation of an aromatic amino acid residue, then changes in CD intensity of this band would indicate an altered protein conformation.

On the other hand, the possibility exists that an ultraviolet heme transition may contribute to the circular dichroism of peroxidase in the 280-m$\mu$ region. Heme does absorb weakly at 280 m$\mu$ (ref. 20) and is known to contribute partly to the 280-m$\mu$ absorption of unpolarized light by hemoglobin[21], myoglobin[21,22] and cytochrome $c$[23]. Thus the heme moiety of peroxidase probably also has weak ultraviolet absorption bands near 280 m$\mu$. Since weak absorption bands frequently have appreciable circular dichroism[10,19], it seems possible that the 282-m$\mu$ CD band of peroxidase may be partly due to a heme chromophore. If this be true, then any change in the heme oxidation state, such as occurs in $E$–$S_I$ and $E$–$S_{II}$, would be expected to affect the CD bands arising from this chromophore.

During peroxidatic activity, the 282-m$\mu$ CD band of peroxidase is altered. This CD band is less intense in $E$–$S_I$ than in either $E$–$S_{II}$ or peroxidase. In view of the above discussion of the 280-m$\mu$ CD bands, it is not possible at present to determine whether

the low intensity of this CD band in $E$–$S_I$ results from a change in the orientation of an aromatic amino acid residue or whether it simply results from a change in an ultraviolet heme absorption band caused by oxidation of the heme.

Another measure of enzyme conformation is provided by the ultraviolet CD bands at 207 and 222 m$\mu$. Since these bands are unaltered by the addition of hydrogen peroxide to peroxidase, the conformation of the peptide backbone in $E$–$S_I$ and in $E$–$S_{II}$ appears to remain the same as that of the free enzyme, within an experimental error of about 5%. This finding suggestst that, if any conformational change occurs during the peroxidatic reaction, it is restricted to a small region involving only a few amino acid residues.

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