Mechanism of Enantioselective Oxygenation of Sulfides Catalyzed by Chloroperoxidase and Horseradish Peroxidase. Spectral Studies and Characterization of Enzyme–Substrate Complexes†

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ABSTRACT: The binding of a series of alkyl aryl sulfides to chloroperoxidase (CPO) and horseradish peroxidase (HRP) has been investigated by optical difference spectroscopy, circular dichroism, paramagnetic NMR spectroscopy, and NMR relaxation measurements. The data are consistent with binding of the sulfides in the distal side of the heme pocket with CPO and near the heme edge with HRP. A linear correlation between the binding constants of para-substituted sulfides to CPO and the Taft σ_I parameter suggests that these substrates act as donors in donor–acceptor complexes involving some residue of the protein chain. Spectral studies during turnover show that high enantioselectivity in the CPO-catalyzed oxidation of sulfides results from a reaction pathway that does not involve the accumulation of compound II enzyme intermediate.

Peroxidases catalyze the oxidation of a wide variety of organic compounds using hydrogen peroxide or other peroxides (Dunford & Stillman, 1976; Frew & Jones, 1984). Generally, the catalytic cycle involves two enzyme intermediates, compounds I and II, which represent states two and one electron more oxidized than the native ferric form and are able to abstract one electron from the substrate to produce a free radical. This species may undergo coupling, disproportionation, or reactions with other substrates or molecular oxygen (Yamazaki, 1977). In a few instances, compound I is apparently reduced directly to the ferric form in a two-electron transfer process (Nakamura et al., 1985), depending upon the nature of the substrate.

Chloroperoxidase (CPO),[1] besides being able to function like other heme-containing peroxidases, is able to catalyze a number of reactions that were believed to be typical of catalases (Thomas et al., 1970; Araiso et al., 1981) and to utilize chloride and bromide ions as donors for halogenation reactions (Hewson & Hager, 1978; Libby et al., 1982). More recently, CPO has been found to be capable of some P-450-type reactions such as the N-dealkylation of alkylamines (Kedderis & Hollenberg, 1983; Kedderis et al., 1986) and the epoxidation of alkenes (Mc Carthy & White, 1983; Ortiz de Montellano et al., 1987). Organic sulfides are also substrates of peroxidases (Kobayashi et al., 1986; Doerge, 1986) in oxygenation reactions that are more typical for P-450 and flavin-dependent monooxygenases (Takata et al., 1983; Fujimori et al., 1990; Cashman et al., 1990; Cashman & Olsen, 1990), but the mechanism of these reactions is not clear. In particular, while there is evidence from ^{18}O-labeling studies that oxygen atom transfer from the peroxide to the sulfide is catalyzed by HRP (Kobayashi et al., 1986), CPO (Kobayashi et al., 1986), and the hydroperoxide-dependent hemoprotein soybean sulfoxidase (Blée & Durst, 1987), it is not clear whether the compound I or II derivative of the various enzymes, or both, acts as oxygen donor and whether single-electron transfer steps are involved in the mechanism, as in the normal peroxidase cycle.

Recent spectral (Perez & Dunford, 1990a) and kinetic (Perez & Dunford, 1990b) studies on HRP showed that both compound I and II can catalyze a one-electron oxidation of the sulfides producing sulfur-cation radical species. This radical species could disproportionate generating a dication, which then could convert to sulfoxide by reaction with water, or react with a hydroxyl radical originating from compound II to form the sulfoxide. The latter pathway could explain the observed oxygen atom transfer from hydrogen peroxide to the sulfide. Evidence for the occurrence of one-electron transfer mechanisms has been obtained for LPO (Doerge, 1986) and soybean sulfoxidase (Blée & Schuber, 1989). In this paper, we address the problem of the mechanism of oxygenation of organic sulfides by CPO by reporting spectral experiments monitoring the changes in the enzyme states during the reaction and optical difference spectra and NMR relaxation measurements showing that for both CPO and HRP

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[1] Abbreviations: CPO, chloroperoxidase; HRP, horseradish peroxidase; P-450, cytochrome P-450; CCP, cytochrome *c* peroxidase; NMR, nuclear magnetic resonance; CD, circular dichroism.

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the sulfides bind near the heme active site. This information is available for the binding of several aromatic donor molecules to HRP (Sakurada et al., 1986; Morishima & Ogawa, 1979; Casella et al., 1991) and LPO (Hosoya et al., 1989; Modi et al., 1989a; Casella et al., 1991) and is reported here for the first time for CPO. The mode of binding of the sulfides to CPO is important to explain the origin of the high enantioselectivity observed in the oxygenation reactions (Colonna et al., 1988, 1990, 1992a).

MATERIALS AND METHODS

Materials. CPO was purchased from Sigma as a suspension in 0.1 M sodium phosphate, pH 4, RZ ~ 1.0. The RZ of CPO did not change by purification of the protein by dialysis against 0.04 M phosphate buffer, pH 5.8, and chromatography on a DEAE-cellulose column with a gradient from 0.04 to 0.1 M phosphate buffer, pH 5.8, as described in the literature (Shahangian & Hager, 1981). HRP (mostly isoenzyme C) was purchased from Sigma as a freeze-dried powder (type VI, RZ 3.2 at pH 7.0). The concentrations of the enzyme solutions were determined spectrophotometrically by using $\epsilon_{400} = 91$ mM^{-1} cm^{-1} for CPO and $\epsilon_{403} = 102$ mM^{-1} cm^{-1} for HRP. The sulfides used in the investigation were prepared according to the methods indicated previously (Colonna et al., 1990). The other reagents were of the highest grade available and were used without further purification. All enzyme solutions were prepared from water purified by a Milli-Q system.

Spectral Measurements. Spectral changes during the reaction of enzyme intermediates and the sulfides were observed using an HP-8452 A single-beam diode-array spectrophotometer. The experiments were performed at low temperature (8 °C, the lowest compatible with problems due to condensation of humidity on the optical cell surfaces), on solutions containing $\sim 0.4 \times 10^{-5}$ M CPO (or HRP) and 10^{-3} M sulfide in acetate buffer, pH 5.0–acetone (3:1 (v/v)) that had been previously incubated at 4 °C in a refrigerator for several min. After the spectrum of the solution was recorded, hydrogen peroxide (5.0×10^{-4}–1.9×10^{-3} M final concentration) was quickly added under stirring and several spectra were taken at 0.3-s intervals in the range 300–500 nm. The sulfides used were methyl *p*-tolyl sulfide, ethyl *p*-tolyl sulfide, isopropyl *p*-tolyl sulfide, and methyl 2-pyridyl sulfide.

Substrate Binding Studies. The equilibrium constants for the binding of sulfides to CPO or HRP were determined from spectrophotometric titrations. The measurements were carried out in a medium of 0.01 M acetate buffer, pH 5.0–acetone (3:1 (v/v) for CPO; 1:1 (v/v) for HRP) by adding microliter amounts of concentrated acetone solutions of the sulfides to the enzyme solution and recording difference spectra of enzyme–substrate vs enzyme after incubation for about 0.5 h at 4 °C (longer incubation times did not produce further spectral changes). All measurements were done at 23 °C.

The binding constants (K) were calculated using double-reciprocal plots of absorbance against substrate concentration as described previously (Casella et al., 1991). The stoichiometry of formation of enzyme–substrate complexes was established by Hill plots as described before (Casella et al., 1991). Rectilinear regression lines were obtained by least-squares fittings using a computer program that takes into account the small volume changes of the solutions following each addition of the titrant. Given the low values of the equilibrium constants, corrections for the actual concentration of unbound ligand in solution were not necessary.

CD spectra of the enzymes and enzyme–substrate complexes were recorded on a Jasco J-500 C dichrograph. The CD data are reported in terms of the molar differential absorption coefficient $\Delta\epsilon$ (M^{-1} cm^{-1}).

NMR Measurements. All proton NMR measurements were performed on a Bruker AC-200 spectrometer using a 5-mm probe. Temperatures were calibrated using a methanol/methanol-d_4 solution and controlled by the variable unity of the spectrometer ensuring a precision of ∓1 °C. The enzyme samples were prepared through concentration by ultrafiltration and repeated exchange with D_2O containing the appropriate deuterated buffer (pH meter readings were uncorrected for isotope effects). Typical concentrations of the enzymes for paramagnetic NMR spectra were 0.5–1 mM. The substrates were added directly in the NMR tube containing the enzyme buffer solution; the heterogeneous mixture was thoroughly stirred and then incubated at 4 °C for a few hours before the spectra were read. At least 10 000 transients were accumulated on a spectral width of 40 kHz using 16K data points. The residual water signal was continuously irradiated for 1 s before the acquisition pulse. A line broadening function of 50 Hz was applied before Fourier transformation. The chemical shifts are referred to the residual HDO signal (4.8 ppm).

Relaxation Rate Measurements. Titrations of the substrates with the enzyme were carried out by adding small volumes of the enzyme solution (~10^{-4} M) to 400 μL of the sulfide solution (10–40 mM) prepared in a 3:1 (v/v) mixture of deuterated 0.1 M acetate buffer, pH 5, and deuterated acetone. The measurements of the longitudinal relaxation times ($T_{1\,obs}$) were performed using the standard nonselective inversion recovery pulse sequence, while the Carr–Purcell–Meilboom–Gill pulse sequence was used to obtain the transverse relaxation times (Martin et al., 1979). The number of variable delays used in each measurement of T_1 and T_2 ranged from 16 to 20. Typically, 4K data points were acquired for each free induction decay curve over a spectral width of 2000 Hz and the repetition time was always at least 5 times the longest relaxation time. The values of $T_{1\,obs}$ and $T_{2\,obs}$ were calculated using the nonlinear least-squares fitting routines of the spectrometer software. The longitudinal and transverse relaxation times observed for the substrates in the presence of a given amount of enzyme can be considered the weighted sum of two components for free and bound substrate:

$$1/T_{i\,obs} = (1/T_{i\,b} - 1/T_{i\,f})E_0/(K_D + S_0) + 1/T_{i\,f} \quad (1)$$

where $i = 1$ or 2, $T_{i\,b}$ is the relaxation time for the substrate bound to the enzyme, and $T_{i\,f}$ is that for free substrate. E_0 and S_0 are the initial concentration of the enzyme and substrate, respectively, and K_D is the dissociation constant for the enzyme–substrate complex. From eq 1, which holds when $S_0 \gg E_0$ (Sakurada et al., 1986), $T_{i\,b}$ can be obtained.

$T_{1\,b}$ and $T_{2\,b}$ are related to the paramagnetic component of the relaxation through the following equation:

$$(T_{i\,b})^{-1} = (T_{i\,D})^{-1} + (T_{i\,M} + \tau_M)^{-1} \quad (2)$$

where τ_M is the lifetime for chemical exchange and $(T_{i\,D})^{-1}$ is the diamagnetic contribution to the relaxation in the bound state. This value has been proved to be negligible for HRP (Modi et al., 1989b), and the same has been assumed for CPO. If τ_M is small enough to be neglected, i.e., the exchange between the free and bound state is fast, $T_{i\,b} = T_{i\,M}$. The values of $T_{i\,M}$ so obtained are generally different for the various substrate protons and are expected to increase with increasing temperature. Under conditions of slow chemical exchange, the contribution of τ_M may be relevant. If it becomes the main contribution, the measurement of $T_{i\,b}$ will give directly

τ_M, which will be the same for all substrate protons. In this case, the values of $T_{i\,b}$ are expected to increase with decreasing temperature.

From the $T_{i\,M}$ values, it is possible to calculate the distances of the substrate protons from the paramagnetic center according to the Solomon (1955) and Bloembergen (1957) theory, considering the dipolar terms only. The contact contribution to the paramagnetic relaxation can be neglected when the substrate is not bound to the metal center, as in the present case. At 4.7 T, when the observed nuclei are protons and in the extreme narrowing conditions ($\omega_I^2\tau_c^2 \ll 1$ and $\omega_S^2\tau_c^2 \gg 1$), as it is usually the case for high-spin hemoproteins, the iron–proton distance is given by

$$r(\text{cm}) = ((8.66 \times 10^{-31})T_{1\,M}\tau_c)^{1/6} \quad (3)$$

or

$$r(\text{cm}) = ((1.03 \times 10^{-30})T_{2\,M}\tau_c)^{1/6} \quad (4)$$

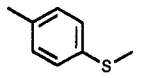
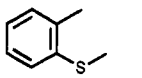
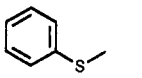
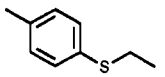
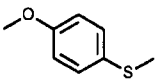
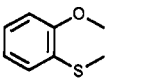
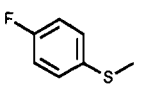
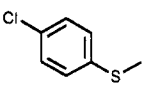
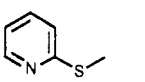
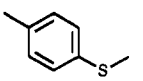
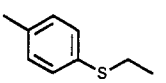
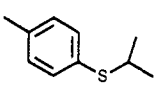
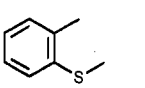
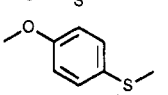
For high-spin hemoproteins of high molecular weight, the dominant contribution to the correlation time is given by τ_S (Sakurada et al., 1986). When the exchange is fast and the relaxation is due solely to dipolar contributions, the value of τ_c (τ_S) can be evaluated by the ratio $T_{1\,M}/T_{2\,M}$ (Dwek et al., 1974). In the present case, this procedure could not be applied since $T_{2\,b}$ appears to be exchange limited (vide infra). An estimate of τ_c was therefore derived from the line width of the heme methyl groups, for which the distance from the ferric ion is known (620 pm).

RESULTS

Optical Spectral Characterization of Enzyme–Sulfide Complexes. In order to obtain some information on the binding of sulfides to native ferric CPO we carried out spectral titrations of the enzyme with several substrate molecules. Due to the extremely low solubility of the sulfides in aqueous medium, the measurements had to be carried out in a solvent mixture of acetate buffer, pH 5.0–acetone (3:1 (v/v)). Higher amounts of acetone were found to lead to some denaturation of the enzyme, as shown by the decrease in intensity of the porphyrin absorption bands, during the time of the binding experiments. Acetate (Sono et al., 1986) and acetone (Sono et al., 1984) are known to bind to CPO, but with extremely low affinity, and actually the optical spectrum of the enzyme in the condition employed here is very similar to that reported in phosphate buffer (Sono et al., 1984). Buffers other than acetate and cosolvents other than acetone were less convenient for this kind of measurement because of their lower ability to keep relatively high concentrations of the sulfides dissolved in the mixed solvent or stronger denaturing effects on the enzyme.

The addition of sulfides to the CPO solution produces some changes in the UV–visible spectrum of the enzyme that can be used for spectral titrations. The full saturation condition, however, could never be achieved. For comparison purposes, sulfide spectral titrations were performed also on HRP in several cases. This enzyme can tolerate higher amounts of acetone so that even the binding of the highly water-insoluble isopropyl *p*-tolyl sulfide could be investigated. The HRP optical spectrum is slightly affected by the presence of acetone, the Soret maximum being shifted from 403 to 405 nm; however, binding of the sulfides produces more significant changes. Analysis of the titration data with double-reciprocal and Hill plots indicates that the observed spectral changes are due to the formation of enzyme–sulfide complexes with 1:1 stoichiometry for both CPO and HRP. The spectral data and apparent binding constants are summarized in Table I.

Table I: Difference Spectra Characteristics and Apparent Binding Constants of CPO–Sulfide Complexes in Acetate Buffer, pH 5–Acetone (3:1 (v/v)) and HRP–Sulfide Complexes in Acetate Buffer, pH 5–Acetone (1:1 (v/v))

donor	spectrum of the complex λ_{min} (nm)	spectrum of the complex λ_{max} (nm)	$\Delta\epsilon$[a] (mM^{-1} cm^{-1})	K (mM^{-1})	n[b]
CPO Complexes					
	400	426	4.3	0.52	0.95
	422	400	17.3	0.11	0.99
	424	400	2.9	0.49	0.95
	424	400	45.2	0.02	1.02
	424	400	8.8	0.32	1.02
	426	402	86.0	0.01	1.10
		400	12.5	0.09	1.06
		402	8.0	0.13	1.06
	424	400	18.0	0.05	1.07
HRP Complexes					
	424	400	6.9	2.23	0.93
	400	424	72.0[c]	0.28	0.99
	400	424	13.0	0.11	1.00
	424	400	25.7	0.23	1.06
	424	400	105.0[c]	0.11	1.03

[a] $\Delta\epsilon$ values were calculated from the extrapolated ΔA_∞ values; $\Delta\epsilon$ represents $|\Delta\epsilon_{peak} - \Delta\epsilon_{trough}|$ for two-signed difference spectra. [b] Hill coefficient. [c] These are overestimated values due to some denaturation of the enzyme during the titration.

In general, the range of K values for the binding of alkyl aryl sulfides to CPO and HRP is similar to that found for the binding of several types of aromatic donor molecules to HRP (Paul & Ohlsson, 1978; Hosoya et al., 1989; Casella et al., 1991). For CPO binding, data of this kind are available for a series of aliphatic thiols, dimethyl sulfide and dimethyl disulfide (Sono et al., 1984), and for a few other nitrogen donor

molecules like imidazole and pyridine (Sono et al., 1986). The aliphatic thiols coordinate in their anionic form to the heme iron producing characteristic hyperporphyrin Soret spectra, while the other sulfur donors bind to a different site (Sono et al., 1984). The affinity of these latter relatively small aliphatic molecules for CPO is generally much lower than that of the alkyl aryl sulfides reported here, but it is apparent from the data in Table I that increasing the size of the sulfur alkyl substituent depresses the affinity for both CPO and HRP. Also, the change in the substitution pattern of the aromatic nucleus from para to ortho and an increase in the electron-withdrawing power of the phenyl ring substituent led to a decrease in affinity for the enzymes. A very good linear correlation between the sulfide binding constants to CPO and the Taft σ_I parameters (Hansch et al., 1991), measuring the field effect of the substituent, for the series of para-substituted phenyl methyl sulfides has been found (slope = –0.775; r = 0.999). Unfortunately, it was not possible to establish a similar relationship in the case of HRP because the affinity of several of the sulfides containing electron-withdrawing substituents for this enzyme was too low to obtain reliable data.

Circular Dichroism Spectra of Enzyme–Sulfide Complexes. Since the CD activity induced in the heme chromophore of CPO and HRP reflects the interaction of the porphyrin electronic transitions with chromophores on nearby residues of the protein (Hsu & Woody, 1971), binding of donor molecules in the heme proximity may affect the CD features of the enzymes. We recorded CD spectra of all the enzyme–sulfide complexes for which binding equilibria were investigated. The changes produced in the CD spectrum of CPO by the presence of excess amounts of the sulfides were modest and rather independent of the identity of the substrate molecules. The CD changes produced by sulfides can be noted between 430 and 480 nm and between 580 and 700 nm. Even though these spectra do not refer to fully saturated conditions, the extent of the CD changes indicates that the presence of a sulfide molecule in the enzyme active site causes little conformational disturbance of the protein residues responsible for the induction of optical activity in the heme chromophore. Somewhat more pronounced are the CD changes produced by the presence of sulfides in the spectrum of HRP throughout the visible spectral range; the extent of the changes increases with the size of the alkyl group bonded to the sulfur atom.

NMR Spectra of Enzyme–Sulfide Complexes. It is known that binding of some aromatic molecules to HRP produces small but detectable changes in the positions of the heme porphyrin signals (Morishima & Ogawa, 1979). Therefore, it was of interest to investigate whether similar effects could be observed here. Paramagnetic ^{1}H NMR spectra of CPO and HRP were thus investigated in the presence of an excess of methyl p-tolyl sulfide or methyl 2-pyridyl sulfide, which exhibit a little solubility in aqueous medium. The sulfides floated over the buffer solution in D_2O, but apparently the rather high enzyme concentration used in these measurements facilitated their diffusion into the buffer medium since some evident changes occur in the proton NMR spectra of the enzymes (Figure 1 and 2).

The NMR spectrum of our CPO sample is similar to those reported previously (Goff et al., 1985; Lukat & Goff, 1986). On addition of the sulfides, some apparent changes are undergone by the upfield signal at –7.4 ppm, attributed to an amino acid side chain close to the heme, the group of signals in the 20–40 ppm region, attributed to porphyrin methylene groups, and the intense heme methyl signals between 40 and

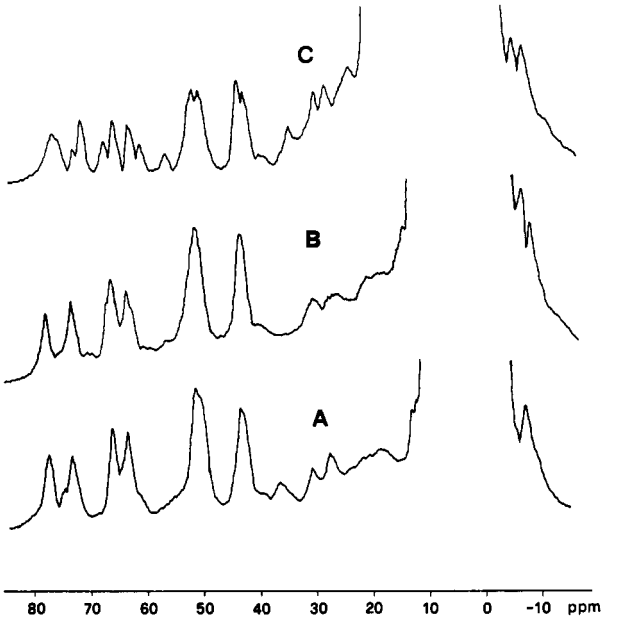


FIGURE 1: Proton NMR spectra of (A) CPO (1 mM, 0.1 M phosphate buffer in D_2O, pD 4.5) and the enzyme after the addition of excess methyl p-tolyl sulfide (B) or methyl 2-pyridyl sulfide (C) and incubation at 4 °C for a few hours.

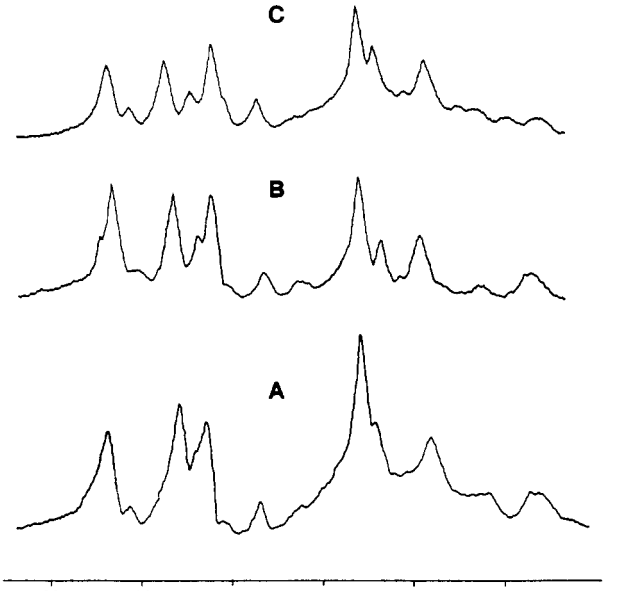


FIGURE 2: Proton NMR spectra of (A) HRP (1.3 mM, 0.1 M phosphate buffer in D_2O, pD 6.5) and the enzyme after the addition of excess methyl p-tolyl sulfide (B) or methyl 2-pyridyl sulfide (C) and incubation at 4 °C for a few hours.

80 ppm (Goff et al., 1985; Lukat & Goff, 1986). The changes in the NMR spectrum of HRP in the presence of the sulfides are less marked than in that of CPO. The middle porphyrin methyl signals near 72 and 76 ppm, which are assigned to the groups in positions 1 and 8 of the porphyrin ring (La Mar et al., 1980), are the most affected. The effects produced by the sulfides on the NMR spectrum of HRP are, however, similar to those observed previously on binding other aromatic donor molecules to the enzyme (Morishima & Ogawa, 1979).

Relaxation Time Measurements. The proton longitudinal (T_1) and transverse (T_2) relaxation times of methyl 2-pyridyl sulfide and methyl p-tolyl sulfide have been measured at 23 °C in the presence of increasing amounts of CPO and HRP. For the system CPO–methyl 2-pyridyl sulfide, the measurements were performed also at 5 °C. Figure 3 shows, for the

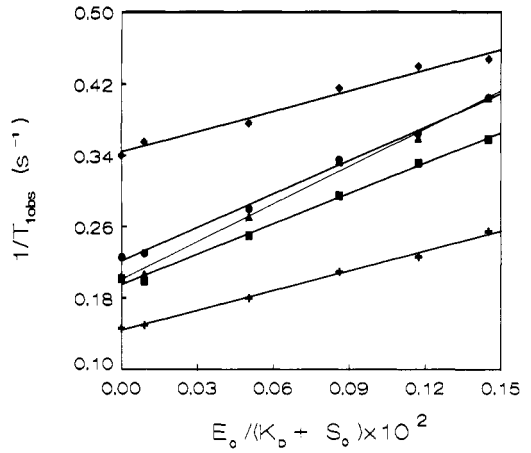


FIGURE 3: Plot of $1/T_{1\,obs}$ vs $E_0/(K_D + S_0)$ for the protons of methyl 2-pyridyl sulfide in the presence of CPO: (■) 2-H, (●) 4-H, (▲) 3-H, (◆) S-CH_3, (+) 1-H (see Table II). The concentration of sulfide varied from 20 to 40 mM and that of CPO from 0 to 0.08 mM in deuterated acetate buffer, pD 5–deuterated acetone.

different proton resonances of the substrate, the experimental results obtained for this system plotted as $T_{1\,obs}^{-1}$ vs $E_0/(K_D + S_0)$. Straight lines have been obtained for all resonances of both substrates, confirming that the binding stoichiometry is 1:1. The calculated values of $T_{1\,b}$ and $T_{2\,b}$ are collected in Table II. Through a least-squares fitting of the T_1 data according to the equation used by Modi et al. (1989c), we obtained binding constants in good agreement with the values determined by spectrophotometric titrations (±30%), although calculations of K_D through T_1 measurements are less accurate for methyl *p*-tolyl sulfide due to its lower solubility.

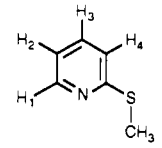
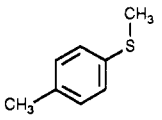
The ^1H NMR spectrum of methyl *p*-tolyl sulfide exhibits only two resolved resonances: one for the aromatic protons, possibly due to accidental degeneracy, and the other for the methyl group bound to sulfur; the *p*-methyl group signal is buried under the signal of acetone. Significantly different values of $T_{1\,b}$ and $T_{2\,b}$ have been obtained for the resolved resonances of the substrate upon interaction with the enzymes, indicating that the relaxation times are not exchange limited. For methyl 2-pyridyl sulfide, all the expected proton resonances are observed. These can be separated in two groups according to their $T_{1\,b}$ values, but the $T_{2\,b}$ values calculated for the interaction with CPO are very similar and suggest that the lifetime in the bound state, τ_M, is not small compared to $T_{2\,M}$. The relaxation rate measurements at 5 °C supported this interpretation since while $T_{1\,b}$ decreases $T_{2\,b}$ remains almost constant. The decrease of $T_{1\,b}$ with temperature is as expected for relaxation rate dominated by $T_{1\,M}^{-1}$, when it holds that $(\tau_c\omega_I)^2 \ll 1$ and the correlation time increases on lowering the temperature. The behavior of $T_{2\,b}$ indicates that dipolar paramagnetic relaxation is not the main contribution to the line width for methyl 2-pyridyl sulfide and it likely results from a balance between the change of τ_M, expected to increase on lowering the temperature, and that of $T_{2\,M}$, for which an opposite trend is expected.

In order to get structural information on the binding, the value of τ_c should be known. To the best of our knowledge, no such value for CPO is available in the literature, while for HRP different values have been used by various authors, i.e., 9.5×10^{-11} s (Modi et al., 1989b) or 5×10^{-11} s (Schejter et al., 1976). Indeed, owing to the $^1/_6$ exponent in eqs 3 and 4, the distance between the substrate and the metal center increases only by 12% on doubling τ_c, so that the general conclusions concerning the binding of the substrate close to the metal center do not change significantly. The ratio $T_{2\,M}/T_{1\,M}$ (Dwek et al., 1974) can be used to calculate τ_c (Modi et al., 1989b,c). This ratio, however, is really sensitive to τ_c when the exchange is fast and the system is outside the extreme narrowing conditions for the observed nucleus (Dwek et al., 1974). The intermediate exchange behavior observed for the binding of methyl 2-pyridyl sulfide to CPO prevents use of the ratio $T_{2\,M}/T_{1\,M}$ for deriving τ_c, and the temperature dependence of $T_{1\,b}$ for this substrate is as predicted under the extreme narrowing conditions. Estimates of τ_c for HRP and CPO were thus evaluated from the line width of the paramagnetically shifted heme methyl proton resonances. The values obtained are 9.9×10^{-11} s for HRP and 8.8×10^{-11} s for CPO. The agreement of the τ_c value found for HRP with those reported previously (Modi et al., 1989b) makes us confident with the τ_c value used for CPO in the calculations of the distances between substrate protons and heme iron (Figure 4). Note that these calculated distances are upper estimates since the Curie contributions to the line width have been neglected. In general, the substrates seem located slightly closer to the heme in CPO complexes than in HRP complexes. However, the iron–proton distances found in the HRP–sulfide complexes are similar to those reported previously for complexes with other aromatic donor molecules (Sakurada et al., 1986; Casella et al., 1991).

Spectral Changes during Turnover. A recent spectral investigation of the reaction of HRP, hydrogen peroxide and *p*-methoxyphenyl methyl sulfide has shown a complete normal peroxidase cycle: first the formation of compound I, followed by formation of compound II, and return to the native enzyme (Perez & Dunford, 1990a). We repeated the same experiment using methyl *p*-tolyl sulfide as a substrate and observed a similar behavior. Experiments of this kind, however, are impossible with CPO in the absence of multimixing, rapid-scan techniques because of the instability of the compound I and compound II derivatives of this enzyme (Lambeir et al., 1987; Rutter et al., 1984). In order to have an idea of the relative importance of CPO compound I and compound II in the oxidation of the sulfides, we monitored the spectral changes undergone by the enzyme during the reaction with several substrates in the presence of various amounts of hydrogen peroxide at low temperature. As discussed above in more detail, the experiments had to be carried out in buffer containing acetone in order to keep the sulfide dissolved in the reaction medium. However, we reported previously that the presence of acetone has a modest effect on the enzymatic conversions of sulfides into sulfoxides (Colonna et al., 1990). As shown in Figure 5 for two separate experiments, the addition of hydrogen peroxide to a mixture of CPO and methyl *p*-tolyl sulfide produces an initial decrease in the absorption spectrum, due to partial formation of compound I, followed by slow recovery of the spectrum of native enzyme and unreacted enzyme–sulfide complex (a significant portion of hydrogen peroxide is consumed in the parallel catalase reaction).

The time course of the various enzyme forms during the reaction can be better appreciated by the absorbance traces at 410 nm for the enzyme–sulfide complex and at 424 nm (the isosbestic point between compound II and CPO) for compound I and at 444 nm (the isosbestic point between compound I and CPO) for compound II (Lambeir et al., 1987). As shown in the inserts of Figure 5, the amount of compound II formed during the oxidation of methyl *p*-tolyl sulfide is very low. When the same experiments were performed with isopropyl *p*-tolyl sulfide, the amount of compound II produced in the reaction was significantly higher (Figure 6), as shown by the absorbance in the 440–450-nm range and the trace at 444

Table II: Relaxation Times for Binding of Methyl 2-Pyridyl Sulfide and Methyl *p*-Tolyl Sulfide to CPO and HRP

substrate	T (K)	proton	CPO		HRP	
			T_{1b} (s)	T_{2b} (s)	T_{1b} (s)	T_{2b} (s)
	295	H_1	1.3×10^{-2}	3.1×10^{-3}	2.3×10^{-2}	6.9×10^{-3}
		H_3	6.3×10^{-3}	3.0×10^{-3}	1.4×10^{-2}	4.5×10^{-3}
		H_4	7.0×10^{-3}	3.0×10^{-3}	1.6×10^{-2}	5.0×10^{-3}
		H_2	8.5×10^{-3}	3.2×10^{-3}	1.3×10^{-2}	4.8×10^{-3}
		S-CH_3	1.6×10^{-2}	4.0×10^{-3}	3.0×10^{-2}	5.2×10^{-3}
	278	H_1	9.0×10^{-3}	3.0×10^{-3}		
		H_3	4.5×10^{-3}	2.7×10^{-3}		
		H_4	4.0×10^{-3}	2.5×10^{-3}		
		H_2	5.0×10^{-3}	3.0×10^{-3}		
		S-CH_3	1.0×10^{-2}	4.0×10^{-3}		
	295	Ph-H	2.2×10^{-2}	4.7×10^{-3}	9.0×10^{-3}	3.1×10^{-3}
		S-CH_3	4.0×10^{-2}	2.0×10^{-2}	1.2×10^{-2}	3.9×10^{-3}

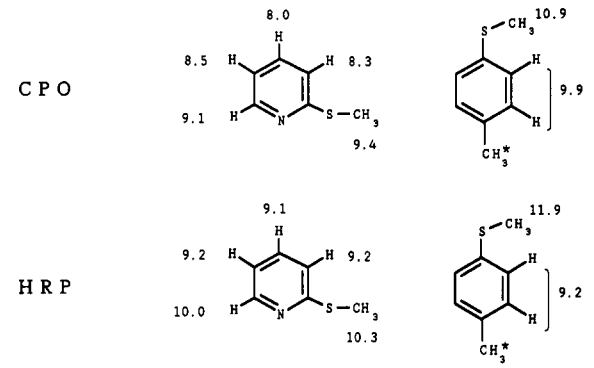


FIGURE 4: Iron–proton distances (Å) for CPO–sulfide and HRP–sulfide complexes derived from relaxation time measurements. The NMR signal of the methyl group marked with an asterisk is buried under the acetone signal.

nm. The behavior of ethyl *p*-tolyl sulfide was somewhat intermediate between those of methyl *p*-tolyl sulfide and isopropyl *p*-tolyl sulfide, whereas the behavior of methyl 2-pyridyl sulfide was very similar to that of methyl *p*-tolyl sulfide (data not shown). By contrast, when these experiments were carried out with HRP, it was clear that compound II is by far the major form of the enzyme during turnover, as shown in Figure 7 for the reaction of methyl *p*-tolyl sulfide.

DISCUSSION

Chloroperoxidase catalyzes the S-oxygenation of alkyl aryl sulfides with remarkable enantioselectivities (Colonna et al., 1990, 1992a). Taking into account the unavoidable minor occurrence of the parallel nonenzymatic sulfide oxidation, in many instances the enantioselectivity is practically absolute. The stereochemical output of the reaction is little dependent on the oxidant used (Colonna et al., 1992a), provided the experimental conditions of the reaction are carefully controlled. It seems, therefore, that the enantioselectivity results from the interaction of sulfides with a chiral environment near the heme active site. We have investigated the binding of a series of alkyl aryl sulfides to native CPO using optical, CD, and NMR spectroscopy and have found that these substrate molecules indeed bind to the enzyme close to the heme group. The mode of binding seems specific, and the CPO active site topology is better fitted by sulfides structurally related to methyl *p*-tolyl sulfide. The steric requirements imposed by the enzyme lead to decreased affinity when the substitution pattern of the aromatic nucleus of the sulfide is changed or

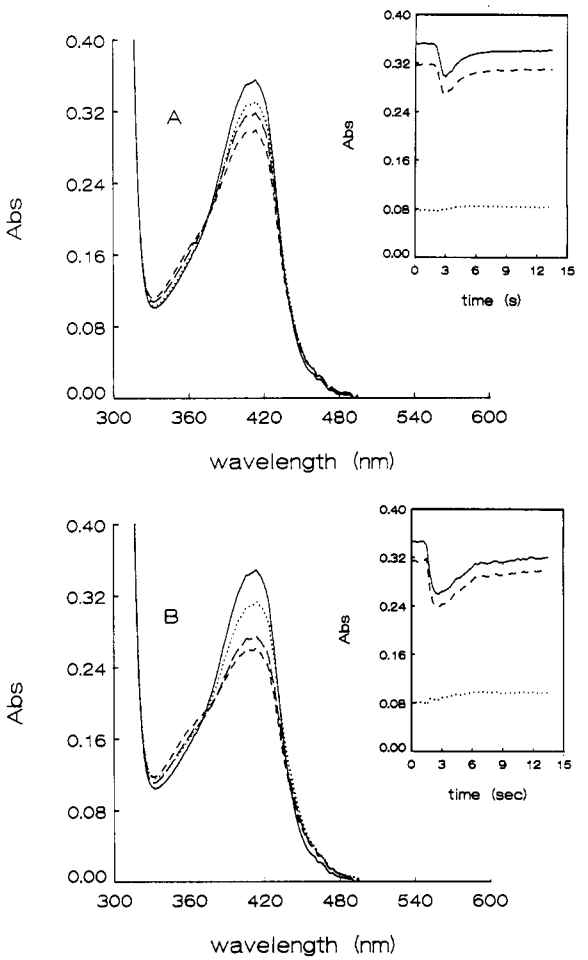


FIGURE 5: Soret spectral changes observed after mixing CPO, methyl *p*-tolyl sulfide, and H_2O_2 in acetate buffer, pH 5–acetone (3:1 (v/v)). (A) Final concentrations: 4.0 μM CPO, 1.0 mM sulfide, and 0.5 mM H_2O_2. Spectra were recorded before addition of hydrogen peroxide (—) and 1.0 (- - -), 1.9 (– –), and 2.8 s (···) after the addition of hydrogen peroxide. (B) Final concentrations: 3.7 μM CPO, 1.0 mM sulfide, and 1.9 mM H_2O_2. Spectra were recorded before the addition of hydrogen peroxide (—) and 1.2 (- - -), 2.7 (– –), and 4.8 s (···) after the addition of hydrogen peroxide. The inserts show the traces of the change in optical absorption at 410 (—), 424 (- - -), and 444 nm (···) with time in the two experiments. The drop in absorbance at 410 nm corresponds to the addition of hydrogen peroxide.

the size of the sulfur alkyl chain is increased. It is significant that these trends in the affinity of sulfides parallels the enantioselectivity pattern observed in the CPO-catalyzed

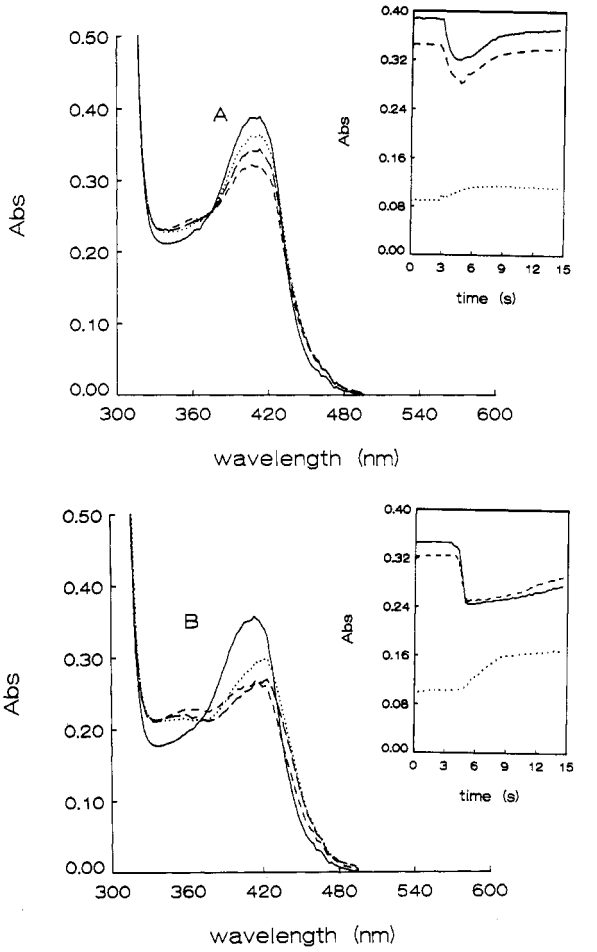


FIGURE 6: Soret spectral changes observed after mixing CPO, isopropyl *p*-tolyl sulfide, and H_2O_2 in acetate buffer, pH 5–acetone (3:1 (v/v)). (A) Final concentration: 4.2 μM CPO, 1.0 mM sulfide, and 0.5 mM H_2O_2. Spectra were recorded before the addition of hydrogen peroxide (—) and 1.8 (- - -), 3.9 (– –), and 6.3 s (···) after the addition of hydrogen peroxide. (B) Final concentrations: 3.7 μM CPO, 1.0 mM sulfide, and 1.9 mM H_2O_2. Spectra were recorded before the addition of hydrogen peroxide (—) and 1.9 (- - -), 5.4 (– –), and 9.6 s (···) after the addition of hydrogen peroxide. The inserts show the traces of the change in optical absorption at 410 (—), 424 (- - -), and 444 nm (···) with time in the two experiments. The drop in absorbance at 410 nm corresponds to the addition of hydrogen peroxide.

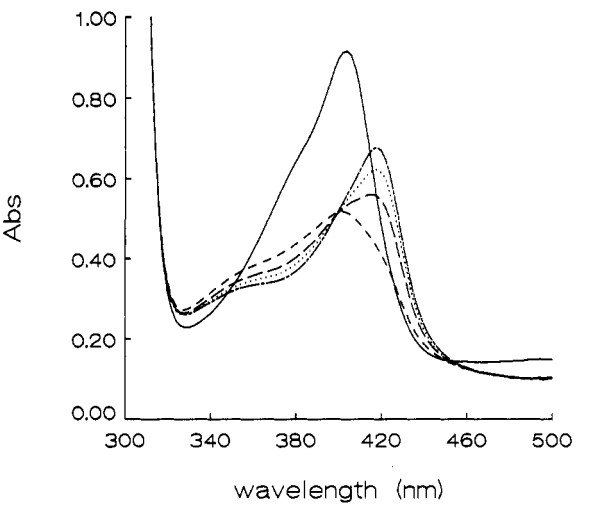


FIGURE 7: Soret spectral changes observed after mixing HRP, methyl *p*-tolyl sulfide, and H_2O_2 in acetate buffer, pH 5–acetone (3:1 (v/v)). Final concentrations: 8.8 μM HRP, 1.0 mM sulfide, and 0.5 mM H_2O_2. Spectra were recorded before the addition of hydrogen peroxide (—) and 1.5 (- - -), 4.8 (– –), 9.3 (···), and 19.5 s (– - –) after the addition of hydrogen peroxide.

oxidation of these molecules (Colonna et al., 1990, 1992a). The present results, therefore, confirm that substrate immobilization by the peptide chain in the active site is the most important parameter controlling the stereochemical aspect of the S-oxygenation reaction. The same picture emerges, on a larger basis, from detailed studies on substrate-dependent parameters, crystallography and site-directed mutagenesis of P-450 enzymes, where the regio- and stereospecificity of monooxygenase reactions are under complete control by the complementary fit between the substrate and enzyme and appropriate positioning of "anchor" groups on the protein backbone near the heme (White et al., 1984; Atkins & Sligar, 1988ab, 1989; Raag & Poulos, 1991; Shimizu et al., 1991).

Unfortunately, the structure of CPO is not known, but we can presume that binding of sulfides occurs on the distal side of the heme pocket. Evidence for the accessibility of this site above the heme comes from recent heme modification studies with phenylhydrazine and sodium azide (Samokyszyn & Ortiz de Montellano, 1991). Binding of the substrate in this position would facilitate the oxygen transfer reaction by the active iron–oxo species, as it occurs in the P-450 enzymes. The linear correlation found between the binding constants of para-substituted sulfides to CPO and σ_I and the negative slope of the resulting plot suggest that these substrates act as donors in donor–acceptor complexes involving some residue of the peptide chain. The interaction giving rise to such complexes may be, for instance, aromatic ring stacking or hydrogen bonding or both. In the former case, the phenyl nucleus of the sulfide would be responsible for the binding interaction, while in the second case the sulfur atom would act as the donor in the complex. Molecular modeling studies on the CPO active site, performed on the basis of the amino acid sequence of the enzyme and homology with CCP (Blanke & Hager, 1990), suggest that a histidine (His 38) and two asparagines (Asn 33 and 37) are located in the distal side of the heme pocket. While the involvement of other residues cannot obviously be excluded, the above three residues are all potential acceptor groups in complexes with the sulfides. In particular, His 38 is an attractive candidate: it is likely to be protonated in the acidic conditions where the enzyme works and may act either as hydrogen-bonding partner or aromatic ring acceptor for the bound sulfide.

Somewhat surprising is perhaps the little difference between the iron–proton distances in the CPO–sulfide complexes and those in the HRP–sulfide complexes (Figure 4), but it should be remembered that these are upper estimates. The binding site of aromatic donor molecules to HRP is near the heme edge (Sakurada et al., 1986; Ortiz de Montellano, 1987). Our NMR experiments indicate that also alkyl aryl sulfides bind to this enzyme in the same position, in the vicinity of the porphyrin methyl groups in position 1 and 8 (Figure 2), as do many phenolic compounds (Sakurada et al., 1986; Casella et al., 1991). The perturbations observed in the NMR spectrum of the heme in CPO on binding the sulfides are more pronounced than in the case of HRP and are apparently distributed among the resonances of all the heme methyl groups (Figure 1). Thus, even if a detailed assignment of the various NMR resonances of the heme in CPO is not available, this result is consistent with binding of the sulfides above the heme plane.

The differences in the mode of binding of sulfides to CPO and HRP are important because they may bear on the different reactivities exhibited by the two enzymes toward this important class of substrates. The catalytic activity of CPO is much

higher than that of HRP; for instance, the k_{cat} value is about 10^4 times higher in the case of thioanisole (Doerge, 1986; Colonna et al., 1992b). Oxygen incorporation from hydrogen peroxide into the substrate is incomplete in the case of HRP, and the extent of incorporation varies depending on the sulfide structure (Kobayashi et al., 1986). The enantioselectivity of the CPO-catalyzed S-oxygenation is very high for all sulfides topographically related to methyl *p*-tolyl sulfide (Colonna et al., 1990, 1992a), while with HRP some enantioselectivity is observed only in three cases (Colonna et al., 1992b). Our spectral studies show that during turnover the relative amount of compound II is much lower than that of compound I in the CPO-catalyzed S-oxygenation (Figure 5), but it is by far the major enzyme species when the same reaction is carried out with HRP (Figure 7). This indicates that the major reaction pathway is probably different for the two enzymes even though, for CPO also, a mechanism involving compound II formation cannot be ruled out.

Spectral and kinetic studies on the oxidation of *p*-methoxyphenyl methyl sulfide by HRP have shown that the reaction occurs according to a competition between a two-step "oxygen rebound" mechanism and another two-step mechanism in which both compound I and compound II react with the sulfide producing cation radicals (Perez & Dunford, 1990a,b). The former is the mechanism proposed for the S-oxygenation catalyzed by P-450 (Watanabe et al., 1981), but we must emphasize that there are important differences in the oxygen rebound mechanism between P-450 and HRP; in the former case compound II is not a real intermediate, since the two-electron transfer steps are very fast, and oxygen delivery from the ferryl oxygen directly to the substrate can occur. Indeed, the actual mechanism of the P-450-mediated S-oxygenation is still not quite clear (Ortiz de Montellano, 1986; Cashman et al., 1990). The proposal is based on correlations between reaction rates and substrate redox potentials (Watanabe et al., 1981). However, the substituent effect found was very small, and also a two-electron mechanism would depend on the substrate oxidation potential. In the case of HRP, binding of the substrate occurs at the heme edge and oxygen incorporation into the sulfide is possible only through a hydroxyl radical released from the ferryl oxygen of compound II (Perez & Dunford, 1990a,b).

The reactivity of CPO in the S-oxygenation is independent of the sulfide oxidation potential (Doerge, 1986), and kinetic studies on a series of aryl methyl sulfides performed by Kobayashi et al. (1987) showed a relatively good correlation between reaction rates and Hammett σ_p parameters. This would agree with a one-step oxygen-transfer mechanism, but the correlation was not considered further because the latter authors observed O-dealkylation in the reactions of 4-alkoxy-substituted thioanisoles (Kobayashi et al., 1987). In our hands, this reaction never occurred appreciably (Colonna et al., 1990, 1992a) and sulfoxide was isolated as the only product. In any case, the activity of CPO in the S-oxygenations occurring with high enantioselectivity can be accounted for by a direct oxygen-transfer mechanism, as proposed recently for the CPO-catalyzed N-oxidation of arylamines (Doerge & Corbett, 1991), or the P-450-like oxygen rebound mechanism. It is interesting that the progressive drop in enantioselectivity observed in the oxidation of ethyl and isopropyl *p*-tolyl sulfide is accompanied by a parallel increase of the importance of compound II in the reaction. This reflects increasing HRP-like behavior in the oxidation and is probably related to the inability of the above substrates to fit properly the active site requirements of CPO in the distal heme pocket. They may be forced to occupy a different site in the protein or confined in the "secondary" active site of CPO which, as in HRP, is located near the heme edge (Samokyszyn & Ortiz de Montellano, 1991).

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