Spectroscopic and binding studies on the stereoselective interaction of tyrosine with horseradish peroxidase and lactoperoxidase

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The interaction of a series of derivatives of tyrosine with horseradish peroxidase (HRP) and lactoperoxidase (LPO) was studied by using optical difference spectroscopy, c.d. and proton n.m.r. spectroscopy in order to reveal differences in the mode of binding of L-tyrosine and D-tyrosine, which are substrates of but react at different rates with the two peroxidases, to HRP and LPO. All the donor molecules form 1:1 complexes with HRP and LPO, but they display a range of affinities for the enzymes. Whereas D-tyrosine binds to HRP more strongly than does L-tyrosine, the opposite holds for the binding to LPO. The distances of the protons of bound tyrosine molecules from the haem iron atoms of HRP and LPO indicate that the site of binding of these substrates is the same as that of simple phenols. This involves the interaction of the phenol nucleus with a protein tyrosine residue [Sakurada, Takahashi & Hosoya (1986) *J. Biol. Chem.* **261**, 9657–9662; Modi, Behere & Mitra (1989) *Biochim. Biophys. Acta* **996**, 214–225]. However, for the present substrates the additional interaction of the carboxylate group with a protein residue (probably an arginine residue) provides further stabilization for the adducts HRP–D-tyrosine and LPO–L-tyrosine with respect to the corresponding complexes with the opposite enantiomers. The differences in the mode of binding of L-tyrosine and D-tyrosine to HRP and LPO is thus determined by the fact that the spatial arrangement of the interacting protein residues can recognize the chirality of the C_(α)–CO₂[−] and C_(β)–C₆H₄OH attachment bonds of the substrates.

INTRODUCTION

Peroxidases (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) are haemoproteins that catalyse the oxidation of a large number of organic and inorganic substrates (Dunford & Stillman, 1976; Morrison & Schonbaum, 1979; Paul & Ohlsson, 1985). Although, in general, peroxidase-catalysed reactions occur with a low degree of specificity for the substrates, the oxidation of the isomers of tyrosine has been reported to occur stereoselectively: horseradish peroxidase (HRP) oxidizes D-tyrosine faster than L-tyrosine, whereas with lactoperoxidase (LPO) the oxidation of the L-isomer occurs more readily (Bayse *et al.*, 1972). The products of the reaction are the isomers of the oxidative coupling dimer *oo'*-dityrosine (Gross & Sizer, 1959; Bayse *et al.*, 1972). Since it is likely that the differences in reactivity of the tyrosine isomers depend on specific interactions between the enzymes and the substrates in the active sites, it is important to obtain information on the spatial disposition of the substrates when bound to HRP or LPO.

Recently n.m.r. (Thanabal *et al.*, 1987*a,b*, 1988*a,b*) and computer modelling studies on HRP (Sakurada *et al.*, 1986), based on sequence similarities to cytochrome *c* peroxidase (Welinder, 1985), for which the X-ray crystal structure is available (Poulos & Kraut, 1980; Finzel *et al.*, 1984), have indicated the nature of several residues near the haem group that are catalytically important for peroxide activation (Arg-38 and His-42) or serve to stabilize the binding of donor molecules in the active site (Arg-183, Tyr-185 and Leu-237). For LPO this kind of information is not available because the protein has a larger size (*M_r* 78000), its primary structure is not known and it contains a very tightly bound haem group, identified as an iron–porphyrin thiol by reductive cleavage with 2-mercaptoethanol (Nichol *et al.*, 1987). These difficulties have so far prevented performance of the

reconstitution experiments with modified haemins that are necessary for the appropriate n.m.r. resonance assignments, although some progress has been made recently (Thanabal & La Mar, 1989).

In the present paper we report on spectroscopic and binding studies on the interaction between a series of derivatives related to tyrosine (Fig. 1) and HRP or LPO. These molecules were selected with the aim of investigating the difference in the mode of binding of L-tyrosine and D-tyrosine to the active sites of the peroxidases. Since it is known that these enzymes have polar protein residues near the haem group (Ortiz de Montellano, 1987; Dawson, 1988), it could be important to know which residues could be involved in the binding of substrates containing several functional groups and the stereochemical requirements that control the interactions with these groups. The LPO-catalysed oxidation of tyrosine may have biosynthetic relevance, because this amino acid is one of the most important potential electron donors in mammalian systems (Bayse *et al.*, 1972).

EXPERIMENTAL

Materials

HRP (mostly isoenzyme C) was purchased from Sigma Chemical Co. as a freeze-dried powder [type VI, RZ (*A*₄₀₃/*A*₂₇₅) 3.2 at pH 7.0]. LPO was purchased from Sigma Chemical Co. as a 3.2 M-(NH₄)₂SO₄ suspension (RZ 0.8 at pH 8.2). The concentrations of the enzyme solutions were determined optically by using ϵ_{403} 102 mm^{−1}·cm^{−1} for HRP and ϵ_{412} 114 mm^{−1}·cm^{−1} for LPO. The other reagents were of analytical grade.

Optical difference spectra and c.d. spectra

Difference spectra (enzyme + substrate versus enzyme) were obtained with an HP 8452 A single-beam diode-array spectro-

Abbreviations used: HRP, horseradish peroxidase; LPO, lactoperoxidase.

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Fig. 1. Structure of the donor molecules investigated

photometer with quartz cells (10 mm path length) at 23 °C. Tyrosine was dissolved in 0.05 M-phosphate buffer, pH 8.2; the other substrates were dissolved in methanol. Titrations were carried out by adding small volumes of concentrated solutions of the substrates to the enzyme solutions. The same amount of substrate solution was then added to a reference cell containing aqueous buffer in order to subtract the absorption of the free substrate, which is significant in the near-u.v. region, and to take into account the variation in solvent composition following each addition of the substrate solution. All spectral titration experiments were made in phosphate buffer at pH 8.2, the pH of maximum enzyme activity for the oxidation of tyrosine (Bayse *et al.*, 1972).

The binding constants (K) were calculated by using the following expression:

$$\frac{1}{\Delta A} = \frac{1}{K \cdot \Delta A_{\infty}} \cdot \frac{1}{[S]} + \frac{1}{\Delta A_{\infty}} \quad (1)$$

where ΔA is the absorbance change caused by a given substrate concentration, ΔA_{∞} is the absorbance change for complete formation of the adduct (at infinite substrate concentration), and $[S]$ is the free substrate concentration, which we assume equal to the initial concentration $[S]_0$ for low-affinity complexes. K and ΔA_{∞} can be evaluated from the slope and intercept of the plot of $1/\Delta A$ versus $1/[S]$. Formation of adducts with 1:1 stoichiometry was confirmed by using the Hill equation in logarithmic form:

$$\log[\Delta A/(\Delta A_{\infty} - \Delta A)] = h \cdot \log[S] + \log K \quad (2)$$

A plot of $\log[\Delta A/(\Delta A_{\infty} - \Delta A)]$ against $\log[S]$ should yield a straight line with slope $h = 1$ in case of single binding of the substrate to the enzyme. Rectilinear regression lines were obtained by least-square fittings with a computer program, taking into account the volume changes of the solutions following each addition of the titrant; the correlation coefficients were all ≥ 0.99 .

C.d. spectra of the enzymes and enzyme-substrate complexes were recorded on a Jasco J-500 C dichrograph, calibrated with a solution of isoandrosterone in dioxan; the c.d. data are reported in terms of molar differential absorption coefficient $\Delta\epsilon$ ($M^{-1} \cdot cm^{-1}$).

Paramagnetic n.m.r. spectra

1H -n.m.r. spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer at 23 °C. 1H_2O in the proteins was exchanged several times with 2H_2O containing 0.05 M-phosphate buffer,

pH 8.2, with the use of an ultrafiltration cell. Solutions of HRP-donor complexes in deuterated buffer were obtained by concentrating the aqueous protein solution to a small volume in an ultrafiltration cell and then adding L-tyrosine or D-tyrosine dissolved in 2H_2O containing 0.05 M-phosphate buffer, pH 8.2. The procedure was repeated several times to remove 1H_2O . Quoted pH values are uncorrected for the small isotope effect. The 1H -n.m.r. spectra were obtained by accumulation of 10000 transients at 16 k data points with a 2 μs pulse. 1H chemical shifts were referred to the signal of trace water. The residual 1H_2O resonance was suppressed during the acquisition time by irradiating the signal at 4.7 p.p.m. at a high power.

T_1 measurements

Longitudinal relaxation-time measurements at various enzyme/substrate molar ratios were carried out by adding 90–200 μl of a solution containing substrate (2.7 mM) and enzyme (25 μM) in 0.05 M-phosphate buffer in 2H_2O to 400 μl of a solution of the substrate (2.7 mM) in the same buffer. The longitudinal relaxation time ($T_{1(obs.)}$) was obtained by the standard inversion-recovery pulse sequence and calculated from the equation:

$$\ln(M_0 - M_z) = -\tau/T_{1(obs.)} + \ln 2M_0 \quad (3)$$

where τ is the time interval between the 180° and 90° pulses, M_z is the z-component of the magnetization, represented by the intensity of the peak at the given τ , and M_0 is the value of M_z when the interval is infinite. M_0 was evaluated from spectra obtained with a very long τ (typically 30 s). $T_{1(obs.)}$ is related to $T_{1,f}$ (relaxation time for free substrate in the bulk solution), $T_{1,b}$ (relaxation time for bound substrate) and K_D (dissociation constant for the enzyme-substrate complex) by the following equation:

$$\frac{1}{T_{1(obs.)}} = \left(\frac{1}{T_{1,b}} - \frac{1}{T_{1,f}} \right) \frac{[E]_0}{K_D + [S]_0} + \frac{1}{T_{1,f}} \quad (4)$$

where $[E]_0$ and $[S]_0$ represent the initial enzyme concentration and initial substrate concentration respectively. This neglects the fraction of bound ligand because the substrate is present in great excess and the free enzyme fraction since all the enzyme present is assumed in the bound form. The K_D values were obtained from the equilibrium constants ($K = 1/K_D$) determined by spectrophotometric titrations. A plot of $1/T_{1(obs.)}$ versus $[E]_0/(K_D + [S]_0)$ enables the values of $T_{1,f}$ and $T_{1,b}$ to be obtained.

The paramagnetic relaxation time ($T_{1,M}$) of bound substrate

Fig. 2. Representative optical difference spectra for the HRP-donor and LPO-donor complexes

(a) HRP (7.4 μM) titrated with 0.4 ml (1), 0.6 ml (2), 0.8 ml (3), 1.0 ml (4), 1.2 ml (5) and 1.4 ml (6) of L-tyrosine solution (5.4 mM). (b) HRP (7.4 μM) titrated with 0.2 ml (1), 0.4 ml (2), 0.6 ml (3), 0.8 ml (4), 1.0 ml (5), 1.2 ml (6) and 1.4 ml (7) of D-tyrosine solution (5.4 mM). (c) LPO (4.4 μM) titrated with 0.2 ml (1), 0.4 ml (2), 0.6 ml (3), 0.8 ml (4), 1.0 ml (5) and 1.2 ml (6) of L-tyrosine solution (5.4 mM). (d) LPO (4.4 μM) titrated with 0.2 ml (1), 0.4 ml (2), 0.6 ml (3), 0.8 ml (4), 1.0 ml (5), 1.2 ml (6) and 1.4 ml (7) of D-tyrosine solution (5.4 mM). All solutions were in 0.05 M-phosphate buffer, pH 8.2. The absorption of free substrates in the near-u.v. region has been subtracted as described in the Experimental section.

resonances can be described by the following equations, including only the dipolar term of the Solomon-Bloembergen equation (Solomon, 1955; Bloembergen, 1957):

$$\frac{1}{T_{1,M}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6} \left(\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2 \tau_c^2} \right) \quad (5)$$

where γ_I , g , β and S have their usual meanings, r is the iron-proton distance, ω_I and ω_s are nuclear and electronic Larmor precession frequencies respectively and τ_c is the correlation time that modulates the electron-nuclear dipolar coupling. For high-spin haemoproteins τ_c can be approximated by τ_s , the electron spin relaxation time (Sakurada *et al.*, 1986). Under conditions of extreme narrowing ($\omega_I^2 \tau_c^2 \ll 1$, $\omega_s^2 \tau_c^2 \gg 1$) (Wüthrich, 1986) the following equation can be obtained (Schejter *et al.*, 1976):

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

For HRP the value $\tau_c = 5 \times 10^{-11}$ s was assumed (Sakurada *et al.*, 1986), giving:

$$r(\text{cm}) = (4.33 \times 10^{-41} T_{1,M})^{\frac{1}{3}} \quad (7)$$

whereas for LPO we used the value $\tau_c = 1 \times 10^{-10}$ s (Modi *et al.*, 1989), giving:

$$r(\text{cm}) = (8.66 \times 10^{-41} T_{1,M})^{\frac{1}{3}} \quad (8)$$

The $T_{1,b}$ values obtained from eqn. (4) were regarded as $T_{1,M}$ values since it is known that the paramagnetic component gives the most significant contribution to $T_{1,b}$ (Sakurada *et al.*, 1986; Modi *et al.*, 1989).

RESULTS

Optical characterization of peroxidase-donor complexes

The addition of excess amounts of the donor molecules reported in Fig. 1 to HRP or LPO produces only small changes

in the Soret and visible-region spectra of the enzymes. Representative optical difference spectra between the enzymes and their adducts with L-tyrosine and D-tyrosine at different degrees of saturation are shown in Fig. 2. The extents of the shift undergone by the Soret bands are similar to those described for the binding of simpler phenolic compounds to HRP (Paul & Ohlsson, 1978; Sakurada *et al.*, 1986; Modi *et al.*, 1989). Treatment of the spectral data according to eqns. (1) and (2) enabled calculation of the binding constants and establishment of the 1:1 stoichiometry for the adducts; the results are presented in Table 1. When compared with the binding constants of other phenols and anilines to HRP (Paul & Ohlsson, 1978; Hosoya *et al.*, 1989), the data in Table 1 show that the presence of a polar alkyl chain increases the affinity for HRP in the case of D-tyrosine and 4-hydroxyphenylpropionic acid, whereas the values of K for the HRP adducts with L-tyrosine, tyramine and (*E*)-4-hydroxycinnamic acid are similar to those of phenol and cresol. Somewhat surprising are the data for LPO-donor complexes, since it is known that simple phenols and anilines have very low affinity for LPO (Hosoya *et al.*, 1989). Here the presence of a polar alkyl chain on the phenolic nucleus increases by about two orders of magnitude the affinity of the donor molecules for LPO; the effect is particularly remarkable for the LPO complexes with L-tyrosine, 4-hydroxyphenylpropionic acid and (*E*)-4-hydroxycinnamic acid.

The small changes produced in the optical spectra of HRP and LPO by binding of the tyrosine derivatives suggest that these molecules do not co-ordinate to the iron(III) centre. This was shown by performing binding experiments of L-tyrosine to the cyanide complexes of HRP and LPO. Cyanide is known to bind with very high affinity to the haem iron of HRP (Arais & Dunford, 1981) and LPO (Dolman *et al.*, 1968); the Soret band for the cyanide derivatives shifts to 422 nm for HRP (Dunford & Stillman, 1976) and to 430 nm for LPO (Carlström, 1969). Titration of the two enzymes with L-tyrosine in the presence of 1.4 mM-CN⁻ shows that binding of the donor molecule occurs without displacement of the co-ordinated anion, since the changes in the Soret spectra of the cyanide adducts are small. The binding

Table 1. Difference-spectra characteristics and apparent binding constants of HRP-donor and LPO-donor complexes in phosphate buffer, pH 8.2

Abbreviations: Tym, tyramine; Hpp, 4-hydroxyphenylpropionic acid; Hci, (*E*)-4-hydroxycinnamic acid. $\Delta\epsilon$ values were calculated from the extrapolated ΔA_{∞} values; $\Delta\epsilon$ represents $|\Delta\epsilon_{\text{peak}} - \Delta\epsilon_{\text{trough}}|$ for two-signed difference spectra.

Enzyme	Donor	Spectrum of the complex (nm)		$\Delta\epsilon$ ($\text{mM}^{-1} \cdot \text{cm}^{-1}$)	K (mM^{-1})	h
		Minimum	Maximum			
HRP	L-Tyr	402		27.3	0.16	1.03
	D-Tyr	394	408	2.3	0.74	1.04
	Tym	396	418	7.1	0.20	0.97
	Hpp		406	4.6	2.73	1.04
	Hci		416	4.0	0.36	1.03
LPO	L-Tyr	412	432	1.1	2.05	1.10
	D-Tyr	412		5.4	0.88	1.08
	Tym		424	3.8	1.34	1.07
	Hpp	410		1.6	2.00	1.04
	Hci		418	1.7	2.78	1.06
HRP-CN ⁻	L-Tyr	422		51.3	0.10	1.01
LPO-CN ⁻	L-Tyr	432	412	8.3	0.83	1.04

constants of HRP-CN⁻-L-tyrosine and LPO-CN⁻-L-tyrosine complexes are lower than but of the same order of magnitude as those of the corresponding complexes of the native enzymes (Table 1). Similar results have been obtained previously for the binding of simple phenols and anilines to the cyanide forms of HRP and LPO (Hosoya *et al.*, 1989).

C.d. spectra of peroxidase-donor complexes

Since the induced haem optical activity in haem proteins depends on the coupling of the haem transitions with transitions located on nearby amino acid residues (Hsu & Woody, 1971), it was of interest to investigate whether binding of the tyrosine derivatives affects the c.d. spectra of the proteins. Although the c.d. spectrum of HRP is very little affected by binding of any of the donor molecules investigated here, that of LPO undergoes significant changes throughout the Soret and visible-region spectral range on binding of L-tyrosine, D-tyrosine or tyramine (Fig. 3). In spite of their high affinity for LPO, the phenolic compounds bearing only the carboxylate functionality in the side chain produce negligible effects on the c.d. spectrum of the enzyme.

N.m.r. spectra and proton relaxation measurements

It has been reported that binding of some aromatic donor molecules to HRP produces under saturation conditions some detectable changes in the chemical shifts of the hyperfine-shifted proton resonances on and near the haem group of the enzyme (Morishima & Ogawa, 1979). We recorded the proton n.m.r. spectra of HRP in the presence of excess of L-tyrosine or D-tyrosine but could not reach the fully donor-molecule-bound condition owing to the limited solubility of the substrates. Binding of the tyrosines affects slightly only the two middle haem methyl signals of HRP at 76.3 and 72.7 p.p.m., which are assigned to the porphyrin methyl groups at positions 1 and 8 (La Mar *et al.*, 1980), and the broader signals in the range between 40 and 50 p.p.m. The corresponding n.m.r. spectra of LPO-donor complexes were not investigated because the assignments of the various haem proton signals are still unavailable.

The presence of HRP or LPO affects the proton resonances of L-tyrosine and D-tyrosine; the absence of separate signals for the bound and free substrates indicates that the ligand exchange is fast on the n.m.r. time scale. Although the shifts of the tyrosine proton signals in the presence of small amounts of the enzymes

were very small, the broadening of the lines was appreciable. Values of $T_{1(\text{obs.})}$ were calculated from 180°-τ-90° pulse sequence, and the corresponding T_{1M} values for the different protons were obtained by least-square fit of the data in eqn. (4) with the use of the equilibrium constants deduced from difference-spectra measurements at the same temperature. The distance between each proton of L-tyrosine and D-tyrosine and the haem iron of HRP and LPO was then calculated according to eqns. (7) and (8) respectively; the results are summarized in Fig. 4.

The distances of the aromatic protons from iron(III) are practically identical in the adducts HRP-L-tyrosine and HRP-D-tyrosine, the values obtained here being very similar to those reported by Sakurada *et al.* (1986) for the adducts formed by HRP with simple phenolic compounds. The proton-iron distances deduced from the analysis of the side-chain α-CH-β-CH₂ multiplets, which show typical ABX patterns (Kainosho & Ajisaka, 1975), are in the same range as for the aromatic protons but indicate some difference in the conformation of bound substrate between HRP-L-tyrosine and HRP-D-tyrosine. In each case there are no significant differences between the relaxation of the two β-CH₂ protons. For the adducts LPO-L-tyrosine and LPO-D-tyrosine also our values for the distances between the aromatic protons and the haem iron are in line with the data available for other LPO-phenol complexes (Modi *et al.*, 1989). In general, all the distances of the protons from the iron for the LPO-tyrosine complexes are larger than those for the HRP-tyrosine complexes. However, we note that the relaxation of the two β-CH₂ protons becomes significantly different in both the LPO-L-tyrosine and the LPO-D-tyrosine complexes, and in both cases it is found that the β-CH proton at higher field lies closer to the iron. Comparing the distances in the two tyrosine adducts, it seems that the whole α-CH-β-CH₂ fragment of LPO-D-tyrosine can approach the iron closer than the corresponding fragment of LPO-L-tyrosine.

DISCUSSION

Aromatic donor molecules bind to HRP (Schejter *et al.*, 1976; Paul & Ohlsson, 1978; Sakurada *et al.*, 1986) and LPO (Hosoya *et al.*, 1989; Modi *et al.*, 1989) near the haem group; the existence of a definite binding site has been confirmed by affinity-chromatography studies on phenyl-Sepharose (Paul & Ohlsson, 1980). For HRP the more advanced computer-aided con-

Fig. 3. C.d. spectra of: (a) HRP (—), HRP in the presence of 510 molar equiv. of L-tyrosine (---) and HRP in the presence of 510 molar equiv. of D-tyrosine (····); (b) LPO (—), LPO in the presence of 740 molar equiv. of L-tyrosine (---), LPO in the presence of 850 molar equiv. of D-tyrosine (····) and LPO in the presence of 425 molar equiv. of tyramine (— · —).

All spectra were recorded in 0.05 M-phosphate buffer, pH 8.2.

formational analysis available (Welinder, 1985) has enabled identification of a cavity surrounded by residues Tyr-185 and Arg-183 and by the 8-methyl group on the haem group in which the aromatic donor molecules can be accommodated (Sakurada *et al.*, 1986). The main contribution to the binding of simple phenolic compounds comes from the aromatic hydrophobic interaction with the nucleus of Tyr-185. Our spectroscopic and relaxation studies indicate that binding of L-tyrosine and D-tyrosine to HRP occurs at the same site as that of the other phenols. In particular the iron-proton distances found for the HRP-tyrosine complexes confirm the nearly perpendicular disposition of the aromatic ring of the substrates with respect to the haem plane. The distances of the protons of the α -CH- β -CH₂ fragments indicate that the polar substituents on the amino acid α -carbon atoms may come closer to the iron than does the aromatic nucleus of the tyrosines. These groups may interact with suitably oriented polar protein residues, providing additional stabilization to the substrate complexes.

From the examination of the binding behaviour of the donor molecules in Fig. 1 to HRP it is clear that, whereas the presence of an amino group on the alkyl chain substituent of the phenol gives no additional stabilization to the HRP-donor complex, a carboxylate group can give marked contribution to complex-formation. We assume that the amino acid residue of HRP that

Fig. 4. Distances (nm) between the various protons of L-tyrosine and D-tyrosine and the haem iron in the complexes HRP-L-tyrosine, HRP-D-tyrosine, LPO-L-tyrosine and LPO-D-tyrosine

The values were calculated as described in the Experimental section.

Fig. 5. Minimum-energy conformers of D-tyrosine and L-tyrosine viewed about the α -C- β -C bond

takes part to this interaction with the carboxylate group is Arg-183. It is apparent, though, that there are rather strict stereochemical requirements for the establishment of such an interaction because the orientation of the carboxylate group *trans* to the phenol group is apparently ineffective. Considering the staggered rotamers in Fig. 5, it is clear that the conformations I of D-tyrosine and IV of L-tyrosine, which are more stable on energy grounds (Inoue *et al.*, 1981), cannot fit the steric requirements for proper interaction with Arg-183 and Tyr-185 at the active site of HRP. The binding of D-tyrosine to the enzyme

is probably preferred with respect to L-tyrosine because for one of the *gauche* rotamers II and III the appropriate juxtaposition between the enzyme and substrate functional groups can be achieved.

For LPO information on the active-site structure is scarce. The presence of distal arginine and histidine residues similar to those of HRP has been proposed on the basis of n.m.r. studies (Shiro & Morishima, 1986; Thanabal & La Mar, 1989). A recent report indicates the possible involvement of a tyrosine residue in the binding of aromatic donor molecules (Hosoya *et al.*, 1989). We find here that the presence of polar groups on the alkyl chain substituent of the phenol increases the affinity of the donor molecules to LPO remarkably; the highest values of the binding constant for the LPO-donor complexes are actually the same as those of the HRP-donor complexes with this type of molecules. Our relaxation studies agree with the view that the mode of binding of aromatic substrates to LPO may be similar to that to HRP (Sakurada *et al.*, 1987; Modi *et al.*, 1989), even though the bound donor molecules are slightly less close to the iron centre of the enzyme. The distance data for the α -CH- β -CH₂ fragments indicate a rather high degree of immobilization of this flexible part of the molecule in the bound substrates. Clearly this is due to the multiple binding interactions available for the tyrosines in the active site of LPO.

As with HRP, the presence of a carboxylate group on the alkyl chain of the substrates provides the main contribution to the stability of the LPO-donor adduct, and therefore we assume that this group can interact with a positively charged amino acid residue, perhaps an arginine residue judged from the similarities of the *K* values observed for the HRP-donor and LPO-donor complexes. Now it is the carboxylate orientation *trans* to the phenol group that is particularly effective for interaction with the protein amino acid residue, and this interaction can only be established for the LPO-L-tyrosine complex. Therefore conformer IV of L-tyrosine is preferentially bound to LPO, and probably the orientation of the amino group in the corresponding *trans*-conformer of D-tyrosine (I) disturbs the establishment of the appropriate interaction between the carboxylate group and the amino acid residue. The c.d. spectra of LPO-substrate complexes seem to support this interpretation, since they show perturbation of the LPO-induced haem optical activity when the substrate carries an amino group. This effect is larger for D-tyrosine than L-tyrosine, and thus suggests a stronger unfavourable interaction by its amino group with enzyme or porphyrin groups.

In conclusion, we have shown that the tyrosine enantiomers bind differently to HRP and LPO. In both cases the isomer that is oxidized faster binds more strongly to the enzyme. The chiral discrimination between L-tyrosine and D-tyrosine exerted by the peroxidases depends upon the relative spatial disposition of an aromatic residue and a positively charged residue near the haem group, probably a tyrosine and an arginine residue in both cases. The origin of the stereoselectivity observed in the oxidation of tyrosine by the peroxidases is therefore probably due to the higher degree of immobilization of the more strongly bound isomer in the enzyme-substrate complex. Assuming, as is likely, that formation of the enzyme reactive intermediates, Compounds I and II, does not modify the binding properties of the substrates

to the resting enzyme, this may facilitate the electron-transfer steps during the catalytic cycle of the enzyme.

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