Haem–Peptide Complexes. Synthesis and Stereoselective Oxidations by Deuterohaemin-L-Phenylalanyl-poly-L-Alanine Complexes

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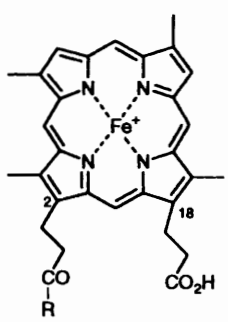
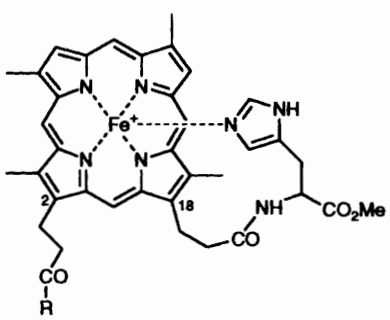
Deuterohaemin-L-histidine methyl ester–peptide complexes have been obtained by covalently linking L-histidine methyl ester and the peptides Ala-Ala-Phe-Ala-Ala-Ala-Ala-Ala-Ala-Ala (compound **3**) or Ala-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Ala-Ala (compound **4**) on the propionic acid side chains. The spectroscopic properties of **3** and **4** and the imidazole binding equilibria indicate that folding of the peptide chain on the opposite part of the porphyrin plane with respect to that occupied by the bound histidine side-arm reduces in the order **3** > **4** the accessibility of exogenous ligands to the sixth co-ordination position of the iron atom, probably through some stacking interactions between the phenylalanine residue of the peptide and the porphyrin ring in the case of **3**. This arrangement has marked consequences on the stereoselectivity observed in a model peroxidase reaction using L- or D-tyrosine methyl ester as substrates and *tert*-butyl hydroperoxide as oxidant in dichloromethane–trifluoroethanol (9:1), that have been interpreted in terms of the interaction between the chiral substrates and the peptide chains of the deuterohaemin complexes.

The haemoproteins are an important class of proteins showing a wide range of biological activities.[1] Among these the oxidative activities exhibited by peroxidase and monooxygenase enzymes have been most extensively studied.[2–4] An important feature of these enzymatic oxidations is they often occur with remarkable selectivity and stereoselectivity. The picture emerging from a variety of physico-chemical or genetic engineering studies on cytochrome P-450[5] and several peroxidases[6] is that substrate binding and immobilization by the peptide chain in the active site, near the haem group, is the most important factor controlling the stereochemical output of the reaction. High stereoselectivities can be achieved when the enzyme active site topology is better fitted by the substrate and suitable 'anchor' groups on the protein backbone are appropriately positioned to interact with the substrate. However, the problem of recognizing specific protein residues involved in the binding of the substrate or even the nature of the interactions stabilizing the enzyme–substrate complexes is, in general, extremely difficult to address unless the three-dimensional structure of the protein is known from X-ray crystallography. In this respect, the investigation of model haem complexes can be very helpful to understand the importance of catalyst–substrate interactions.

We have recently described the synthesis and catalytic activity of the haemin complexes **1** and **2** obtained by covalently linking a peptide residue to deuterohaemin [(3,7,12,17-tetramethylporphyrin-2,18-dipropionato)iron(III)].[7] The folding of these peptide residues should provide an environment to the haem centre that resembles that experienced by this group in the proteins. In the catalytic oxidations of L- and D-tyrosine by hydrogen peroxide, a typical peroxidase reaction,[8] the complexes **1** and **2** exhibited some stereoselectivity attributable to interactions between substrate and peptide chains. Since these interactions may involve either the polar groups or the phenol nucleus of the substrates we thought that a more rational modification of the peptide residue linked to the haemin catalyst could enable to rationalize and hopefully optimize the stereoselectivity in the tyrosine oxidation compatible with the flexibility of the propionic acid arm carrying the peptide chain in these models. To this end we have prepared the



1: R = Ala-Phe-Ser-Phe-Glu-Ala-Gln-Gly-Gly-Leu-Ala
2: R = Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala



3: R = Ala-Ala-Phe-Ala-Ala-Ala-Ala-Ala-Ala-Ala
4: R = Ala-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Ala-Ala
5: R = OH

two new deuterohaemin–peptide complexes **3** and **4** in which a single L-phenylalanine residue is included in a specific position of a poly-L-alanine backbone and, further to improve the models, a L-histidine residue is linked to the other propionic acid side-chain of deuterohaemin; this will provide an intramolecularly bound imidazole ligand to iron(III), that is expected to enhance the catalytic efficiency of the system.[9]

Experimental

Materials and Instrumentation.—Compounds accessible from commercial sources were of the highest purity available and used as received. Dimethylformamide (dmf) was purified by treatment with barium oxide and distilled from calcium hydride under reduced pressure. Deuterohaemin was prepared from haemin according to a literature method.[10] Samples of the Fe^{II}–CN^- derivatives of the complexes for NMR studies were prepared by reducing the Fe^{III}–CN^- species by means of a degassed sodium dithionite solution in deuteriated water, under an inert atmosphere. Optical absorption spectra were recorded with a HP 8452A diode-array spectrophotometer, circular dichroism (CD) spectra on a JASCO J-500 C dichrograph, NMR spectra on a Bruker AC200 spectrometer operating at 200 MHz and high-performance liquid chromatography (HPLC) on a LKB instrument.

Preparation of Ala-Ala-Phe-Ala-Ala-Ala-Ala-Ala-Ala-Ala by Solid-phase Synthesis.—Solid-phase synthesis of this peptide has been carried out on a Biolinx Plus automatic synthetizer, using a polyacrylamide resin as support and dmf as solvent. The amino acids were activated at the carboxylic group by pentafluorophenyl esters and protected at the amino groups with fluorenylmethoxycarbonyl residues. The deprotection steps were monitored following the characteristic fluorenylmethyl residue UV absorption at 304 nm, whereas the coupling steps were monitored by an anionic dye at 600 nm. The peptide was cleaved from the resin by treatment with 95% aqueous trifluoroacetic acid for 1.5 h, using a standard literature method.[11] The purity of the product was tested by HPLC analysis and fast atom bombardment (FAB) mass spectrometry.

Preparation of Ala-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Ala-Ala by Liquid-phase Method.—The reagent Z-(L-Ala)$_3$–OBu^t[7b] (Z = *N*-benzyloxycarbonyl) was unprotected either at the amino group (4.75 mmol) or at the carboxylic group (4.75 mmol) by the general procedures reported below. The carboxylic unprotected tripeptide was dissolved in anhydrous dmf, then 1-hydroxybenzotriazole (14.35 mmol) and, after 0.5 h, dicyclohexylcarbodiimide (4.86 mmol) was added with stirring at 0 °C. After the mixture had been allowed to react for an additional 1 h, the amino unprotected peptide and 1 equivalent of triethylamine were added and stirring was continued for 2 d at room temperature. The dicyclohexylurea thus formed was filtered off and washed with anhydrous dmf. The product Z-(L-Ala)$_6$–OBu^t was unprotected at the amino group and allowed to react in anhydrous dmf with Z-L-Phe-OC_6F_5 overnight at 0 °C. The reaction was followed by TLC using a mixture of chloroform, acetic acid and hexane (8:1:1) as eluent. The product was precipitated by addition of a triple volume of water, filtered off and washed with water and diethyl ether. The heptapeptide Z-L-Phe-(L-Ala)$_6$–OBu^t was unprotected at the amino group by hydrogenolysis as described below and then allowed to react with the carboxylic unprotected Z-(L-Ala)$_3$–OH using the same procedure used to prepare Z-(L-Ala)$_6$–OBu^t. The deprotected decapeptide obtained showed a single HPLC band with a retention time of 12 min when chromatographed on a Viosfer C18 (25 cm × 10 mm) column using a linear gradient (0–60% MeCN in a solution of 0.01% aqueous CF_3CO_2H over 40 min). The FAB mass spectrum was also consistent with the molecular weight and sequence of the desired peptide. The decapeptide was unprotected at the amino group and coupled to the deuterohaemin–histidine as described below.

Removal of the N-*Benzyloxycarbonyl Group* (Z) *from the Protected Peptides.*—The protected peptide (1 mmol) was dissolved in 80% acetic acid (50 cm^3). Palladium–charcoal (10% palladium content) (1 g) was added and the mixture was hydrogenated at atmospheric pressure with stirring, until carbon dioxide evolution ceased. The catalyst was removed by filtration and the filtrate was evaporated to dryness under vacuum. The residue was triturated with diethyl ether, filtered off, washed several times with diethyl ether and dried under vacuum over potassium hydroxide.

Removal of the tert-*Butyl Group from the Protected Peptide.*—The protected peptide was treated with the minimum amount of trifluoroacetic acid and allowed to react for 50 min at room temperature. The solution was taken to dryness under vacuum, treated with diethyl ether, filtered off, washed several times with ether, and dried under vacuum over potassium hydroxide.

*Preparation of Deuterohaemin-2(18)-*L*-Histidine Methyl ester,* **5**.—This compound was prepared by modification of the literature procedure.[12a] Deuterohaemin chloride (1 mmol) was dissolved in anhydrous dmf. Under stirring at 0 °C 1-hydroxybenzotriazole (3 mmol) and, after 0.5 h, dicyclohexylcarbodiimide (1 mmol) were added; the mixture was allowed to stir at 0 °C for an additional 1 h. Then L-histidine methyl ester (1 mmol) and triethylamine (1 mmol) were added and the mixture was allowed to react at 0 °C for 4 h, followed by 20 h at room temperature. The precipitate of dicyclohexylurea was filtered off and the crude product was precipitated by addition of diethyl ether. The product was chromatographed on a silica gel column (4 × 30 cm) by eluting with butanol–acetic acid–water (4:1:1 v/v/v). The eluted fractions consisted of unreacted deuterohaemin, deuterohaemin-L-histidine methyl ester and the product of bis condensation, deuterohaemin-2,18-bis(L-histidine methyl ester), respectively. The desired product was recovered by evaporating to dryness the solution under vacuum; its electronic and CD spectra were identical with those of a sample prepared according to the literature.[12]

Preparation of Complexes **3** *and* **4**.—Deuterohaemin-L-histidine methyl ester (2 mmol) was dissolved in anhydrous dmf. Under stirring at 0 °C 1-hydroxybenzotriazole (6 mmol) and, after 0.5 h, dicyclohexylcarbodiimide (2 mmol) were added; the mixture was allowed to stir at 0 °C for 1 h. Then the carboxyl-protected peptide (1 mmol) and a small amount of lithium chloride, used to increase the solubility of the peptide,[13] were added and the mixture was allowed to react at 45 °C for 2 h, followed by 3 d at room temperature. The solution was reduced to a small volume under vacuum and the product precipitated with diethyl ether. The precipitate was filtered off and washed with water, in order to eliminate LiCl and the unreacted peptide, and then with methanol, to eliminate the unreacted deuterohaemin-L-histidine methyl ester. The deuterohaemin–peptide complexes **3** and **4** showed a single TLC spot [silica gel, butanol–acetic acid–water (4:1:1 v/v/v)] with no trace of the starting materials and were characterized by UV, CD and NMR spectroscopy.

Ligand Binding.—The equilibrium constants for ligand–adduct formation were determined by spectrophotometric titrations at 25 °C. The data were analysed as described previously.[7]

Kinetics.—Kinetic experiments on *p*-cresol oxidation at fixed substrate concentration and variable hydrogen peroxide concentration were carried out as follows. The reaction mixture contained 1.88×10^{-6} mol dm^{-3} complex **4**, 5×10^{-3} mol dm^{-3} *p*-cresol and $(2.5–100) \times 10^{-4}$ mol dm^{-3} hydrogen

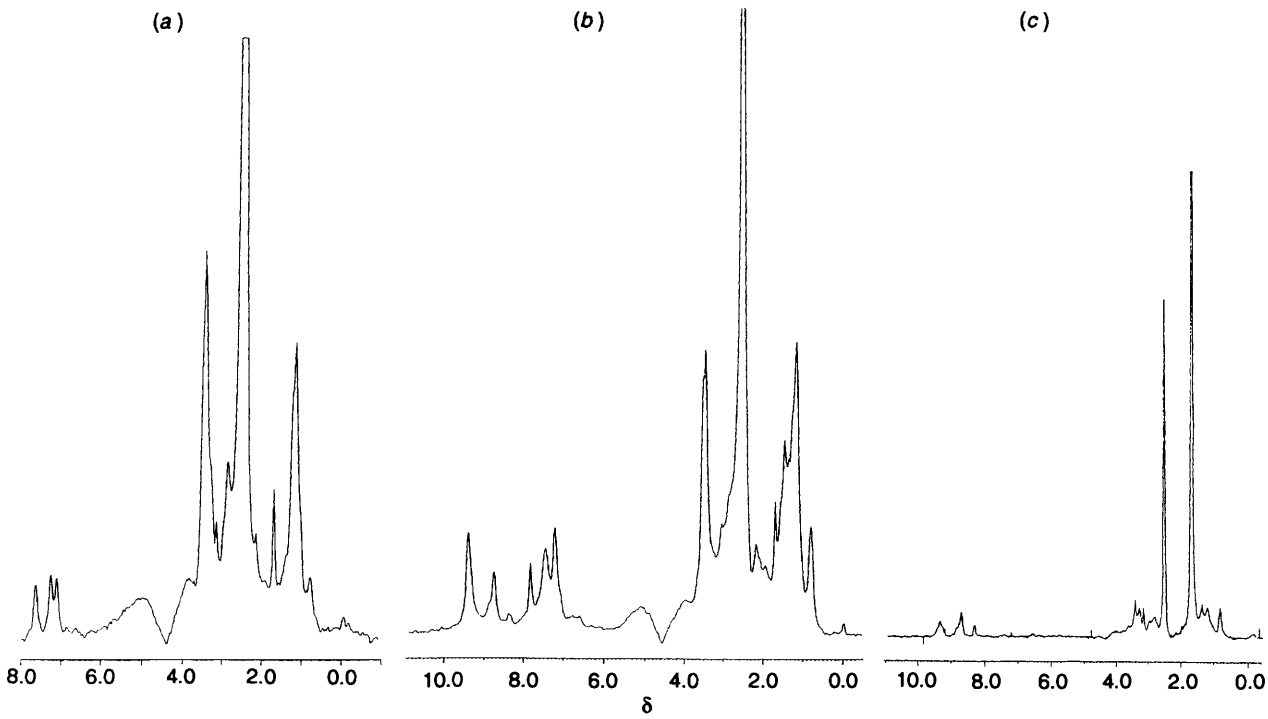


Fig. 1 The NMR spectra of the diamagnetic Fe^{II}–CN^- derivatives of compounds **3** (*a*), **4** (*b*) and **5** (*c*) in $(CD_3)_2SO$–D_2O

peroxide in 0.01 mol dm^{-3} borate buffer at pH 9.0. Initial rates were calculated using the difference in molar absorption coefficients between the mixture of oxidation products and *p*-cresol at 300 nm ($\Delta\varepsilon$ 2350 dm^3 mol^{-1} cm^{-1}).[14] The comparative kinetics of oxidation of *p*-cresol by hydrogen peroxide and compounds **1** and **4** at saturated substrate concentration was studied in the same apparatus employed previously.[7] The reaction mixtures contained 4×10^{-7} mol dm^{-3} of complex **1** or **4**, 1×10^{-3} mol dm^{-3} of oxidant and 6.3×10^{-3} mol dm^{-3} of *p*-cresol in trifluoroethanol(tfe)–CH_2Cl_2 (1:9).

The kinetics of oxidation of L- and D-tyrosine methyl ester by *tert*-butyl hydroperoxide and compounds **3** and **4** at variable substrate concentrations was followed in thermostatted cells equipped with a magnetic stirrer at 20 ± 0.1 °C. The reaction mixtures contained 0.8×10^{-6} mol dm^{-3} of complex **3** or 1.32×10^{-6} mol dm^{-3} of complex **4**, 1.09×10^{-2} mol dm^{-3} of oxidant, and $(1.53–153) \times 10^{-4}$ mol dm^{-3} L- or D-tyrosine methyl esters in tfe–CH_2Cl_2 (1:9). The reactions were initiated by the addition of the oxidant and followed by the increase of the absorption band at 315 nm due to the formation of *o,o'*-dityrosine. Initial rates were calculated using the difference in molar absorption coefficients between dityrosine and tyrosine at 315 nm ($\Delta\varepsilon$ 7300 dm^3 mol^{-1} cm^{-1}). Haemin destruction was negligible within the time used for rate determination. The kinetics of oxidation of L- or D-tyrosine by H_2O_2 and compounds **3** or **4** at variable substrate concentration was followed for reaction mixtures containing 1.3×10^{-7} mol dm^{-3} of catalyst, 1×10^{-3} mol dm^{-3} of oxidant and $(2–300) \times 10^{-3}$ mol dm^{-3} of substrate.

Results and Discussion

The procedure employing 1-hydroxybenzotriazole and dicyclohexylcarbodiimide has proved a convenient method for covalent modification of deuterohaemins through the propionic acid side chains. The condensation of peptides to the free propionic acid residue of deuterohaemin-L-histidine methyl ester required much longer coupling time but also proceeds satisfactorily. The deuterohaemin–histidine–peptide complexes obtained, **3** and **4**, are slightly soluble in alkaline buffer and in solvents like dimethyl sulfoxide or trifluoroethanol. Characterization of the peptide fragments of **3** and **4** can be obtained by the proton NMR spectra of the diamagnetic Fe^{II}–CN^- derivatives of the complexes, shown in Fig. 1. The intense resonances clustered around δ 1.3 identify the methyl groups of the alanine residues, while the aromatic signals of the phenylalanine residue occur in the aromatic region together with those of the pyrrole and *meso* protons of the porphyrin ring, the histidine imidazole protons and other broader signals attributable to peptide NH groups.

Although it is clearly impossible to make a detailed assignment of the various signals in the aromatic region at this stage we note that the pattern for **3** and **4** is different and that the spectrum of the Fe^{II}–CN^- derivative of **5**, lacking the phenylalanine residue, is much simpler in this range, where it exhibits only three signals. One further problem in the assignment is that each of the deuterohaemin complexes obtained by covalent modification at one of the propionic acid side chains is actually an equimolar mixture of the isomers substituted at positions 2 and 18 of the porphyrin ring. This is most easily seen in the NMR spectra of the low-spin Fe^{III}–CN^- derivatives of the complexes, one of which is shown in Fig. 2. These spectra show seven signals (one of which with double intensity) between δ 10 and 20 attributable to the four porphyrin methyl groups of the two isomers. The pattern of these signals is similar in the spectrum of the Fe^{III}–CN^- derivative of **5**. All these spectra show characteristic upfield signals near δ −18 attributable to the pyrrole protons.[15] The NMR spectra of the high-spin iron(III) species **3** and **4** display broader resonances, with larger paramagnetic shifts; easily recognized are, however, the porphyrin methyl signals occurring in the range δ 60–70 (see *e.g.* Fig. 2).

The electronic spectral data of **3** and **4** are summarized in Table 1. In general, the spectra show solvent dependence because of the tendency of porphyrins and non-polar peptides to associate in aqueous media. It is possible that in the alkaline buffer solution used the aggregation involves formation of μ-oxo bridged dimers as this has been observed in other cases.[16] Unfortunately, the insolubility of these haem–peptide complexes at lower pH prevents a detailed study of the equilibria between aqua, hydroxo and μ-oxo species that likely occur in aqueous solution. In the less polar solvents the Soret

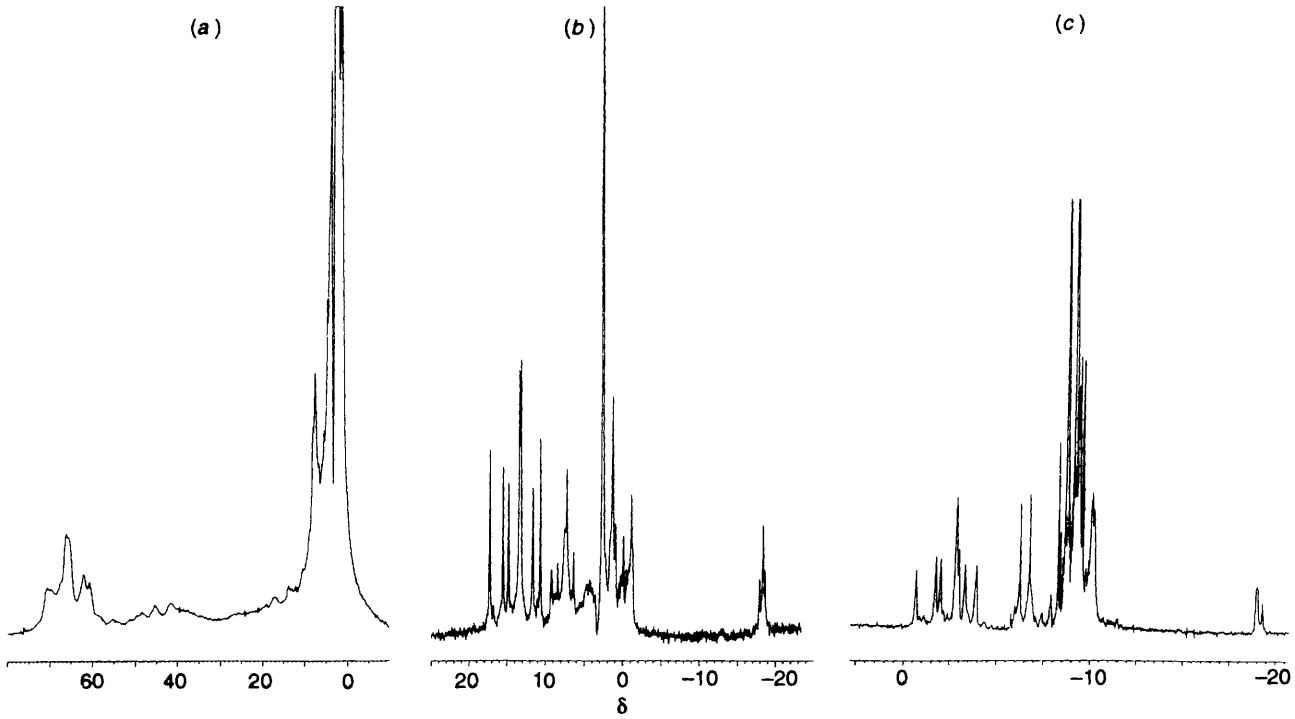


Fig. 2 The NMR spectra in $(CD_3)_2SO$–D_2O of compound **4**: (*a*) high-spin iron(III) species; (*b*) low-spin Fe^{III}–CN^- derivative. The spectrum of the low spin Fe^{III}–CN^- derivative of **5** (*c*) is also shown for comparison

Table 1 Electronic and CD spectral data of deuterohaemin complexes **3**–**5**

		UV/VIS λ_{max}/nm			CD	
Compound	Solvent	Soret	β	α	λ_{max}/nm	($\Delta\varepsilon$/dm^3 mol^{-1} cm^{-1})
3	Borate buffer pH 9.0	392	490	596	345 (+0.6) 425 (−0.05)	400 (+8.0)
	CH_2Cl_2–tfe (9:1)	392	492	610	365 (−0.75)	402 (+2.1)
4	Borate buffer pH 9.0	388	490	595	330 (sh) (−1.0) 413 (−1.2)	380 (−3.25)
	CH_2Cl_2–tfe (9:1)	392	494	61	338 (+0.9)	394 (+0.6)
5	Borate buffer pH 9.0	388	492	594	335 (+1.3)	377 (+2.25)
	Methanol	390	486	582	381 (+2.2)	

band is sharper, suggesting the predominance of the monomeric forms, at least at low concentrations, and in fact solutions of the complexes in tfa–CH_2Cl_2 follow Beer's law in the concentration range 5×10^{-7}–3×10^{-5} mol dm^{-3}. Since the complexes are chiral, CD spectroscopy can be helpful for their characterization. Interestingly, the CD spectra of **3** and **4** are very different from each other (Table 1) and both are different from the CD spectrum of **5**, indicating that the covalent peptide modification of the porphyrin side chain affects the haem chromophore. Although the spectra in aqueous buffer reflect to some extent the existence of associative equilibria, it is noteworthy that the spectrum of complex **3**, containing the phenylalanine residue closer to the amide terminal end of the peptide chain, exhibits optical activity systematically more intense with respect to **4**. We believe that the more intense CD activity reflects reduced mobility of the peptide fragment of **3** near the amide terminal end through some interaction between the phenyl nucleus of the phenylalanine residue in the third position and the porphyrin ring, as the folding of the peptide chain shown by the ligand binding studies suggests.

In order to assess whether the binding of the histidine side chain to iron(III) is able to produce a folding in the peptide side chain of **3** and **4** such that the steric accessibility of the sixth axial position is reduced, we performed spectrophotometric titration experiments of **3**, **4** and **5** in different solvents. In our previous work with **1** and **2** we found that the binding of one imidazole indeed caused significant reduction of the affinity for a second imidazole in the case of **1**, while for **2** this effect was negligible and two imidazoles bound to iron(III) in a single step.[7] The strong tendency to form the bis adducts by the haemins is due to the formation of stable low-spin iron(III) species. For **3** and **4** the fifth co-ordination position is occupied by the histidine imidazole and the eventual folding of the peptide chain towards the opposite side of the porphyrin plane can be established by comparisons of the affinity of these complexes towards an exogenous imidazole with that of **5**. Well behaved spectral titration curves of **3**–**5** with imidazole in different solvents, with several isosbestic points, were obtained in all cases. One example is shown in Fig. 3 as difference spectra. All the final spectra were typical of deuterohaemin–bis(imidazole) complexes, with Soret maximum at ≈400 nm and visible maximum near 540 nm. The binding costants K and Hill coefficients (*i.e.* the stoichiometry of binding) obtained in the titrations are reported in Table 2. Analysing the data it is possible to see that in general the K values are remarkably low in the aqueous solvent, where the reagents are strongly solvated,

Table 2 Binding costants (K/dm^3 mol^{-1}) and Hill coefficients (n) obtained from imidazole titrations in different solvents

	Borate buffer pH 9.0		tfe		tfe–CH_2Cl_2 (1:9)	
Complex	K	n	K	n	K	n
3	64	1.07	5000	1.01	10^6	0.98
4	95	1.00	6200	0.94	10^6	1.02
5	150	1.02	7600	1.02	$>10^7$	1.00

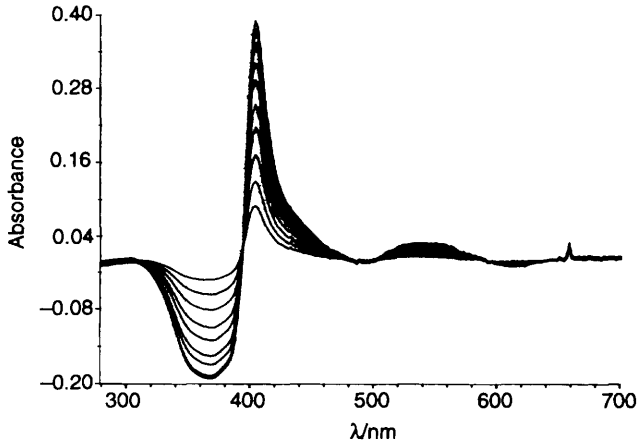


Fig. 3 Titration of a 6.7 × 10^{-6} mol dm^{-3} solution of compound **3** in CH_2Cl_2–tfe (9:1) with a 1 × 10^{-2} mol dm^{-3} solution of imidazole (Him) in CH_2Cl_2–tfe (9:1) (cell path length 1.0 cm). Selected differential spectra show the changes after additions of the titrant corresponding to [Fe]:[Him] ratios of 1:2; 1:4; 1:8; 1:15; 1:25; 1:50; 1:100; 1:300; 1:500; 1:700; 1:900; 1:1000 (12 curves out of 40 studied)

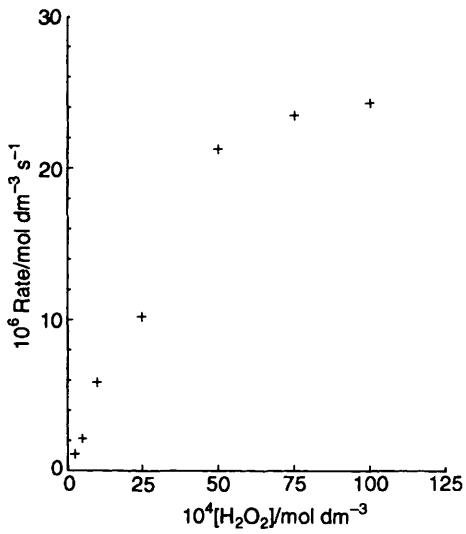


Fig. 4 Dependence on hydrogen peroxide concentration of the initial rates of *p*-cresol oxidation catalysed by **4** in borate buffer, pH 9.0

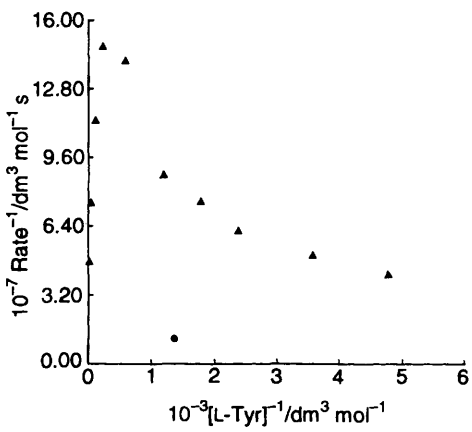


Fig. 5 Dependence on L-tyrosine concentration of the initial rates of dityrosine formation in the oxidation by hydrogen peroxide catalysed by compound **3** in borate buffer, pH 9.0

and progressively increase as the polarity of the medium is decreased. However it is also clear that the affinity for imidazole decreases in the order **5** > **4** > **3**. If we compare the behaviour of **3** and **4** with that of **1** and **2** we have to conclude that it is the presence of the aromatic Phe residue in the peptide chain which is responsible for the steric disturbance caused to the approach of the incoming ligand. This effect is stronger when the position of the Phe residue is closer to the amino terminal end of the peptide chain. It is likely that the phenyl nucleus in this position is better suited to be engaged in ring-stacking interactions with the porphyrin ring.

The possibility to impose some folding of the peptide chain towards the side of the porphyrin plane opposite to the axial ligand raised the idea that this conformational arrangement might be exploited for interaction with substrate molecules in a catalytic reaction. The presence of bulky peptide residues in the modified deuterohaemin complexes **1**–**4**, in fact, increases remarkably the stability to oxidation of the porphyrins by strong oxidizing agents such as hydrogen peroxide, so that these systems can be studied as catalysts in biomimetic oxidation reactions. Comparative experiments performed with **1** and **3** showed that the latter complex was ten times more effective as catalyst in the oxidation of *p*-cresol by hydrogen peroxide by virtue of the presence of the covalently bound imidazole axial ligand. As a suitable reaction to investigate stereoselective effects we chose to study in more detail the peroxidative oxidation of L- and D-tyrosine, as we did with **1** and **2**.[7] In the initial stages of the reaction the product is the dimer *o*,*o*′-dityrosine [α,α′-diamino-6,6′-dihydroxy-1,1′-biphenyl-3,3′-dipropanoic acid], resulting from coupling of phenoxy radicals.[8] On longer reaction times mixtures of products from further polymerization/oxidation reactions are formed.

Compounds **3** and **4**, as well as **1** and **2**,[7] are also active in the catalytic decomposition of hydrogen peroxide. This behaviour has not been investigated in detail because of the competitive destruction of the porphyrin. However, the catalytic activity is depressed in the presence of electron-donor substrates, indicating that the catalytic oxidation of these molecules can effectively compete with decomposition of the oxidant. In the investigation of the catalytic oxidations of phenolic substrates by **3** and **4** we had therefore to search for conditions in which hydrogen peroxide was saturating. This was done by a series of experiments in which the rate of oxidation of the substrate was studied as a function of hydrogen peroxide concentration, until the rate did not increase further on increasing the concentration of H_2O_2. Fig. 4 shows one of these experiments using *p*-cresol as donor substrate.

By studying the dependence of the initial rate of oxidation of L- and D-tyrosine by **3** or **4** and hydrogen peroxide in aqueous borate buffer at pH 9 as a function of the substrate concentration we found that after an initial steep increase the rate undergoes a progressive decrease, with a behaviour which

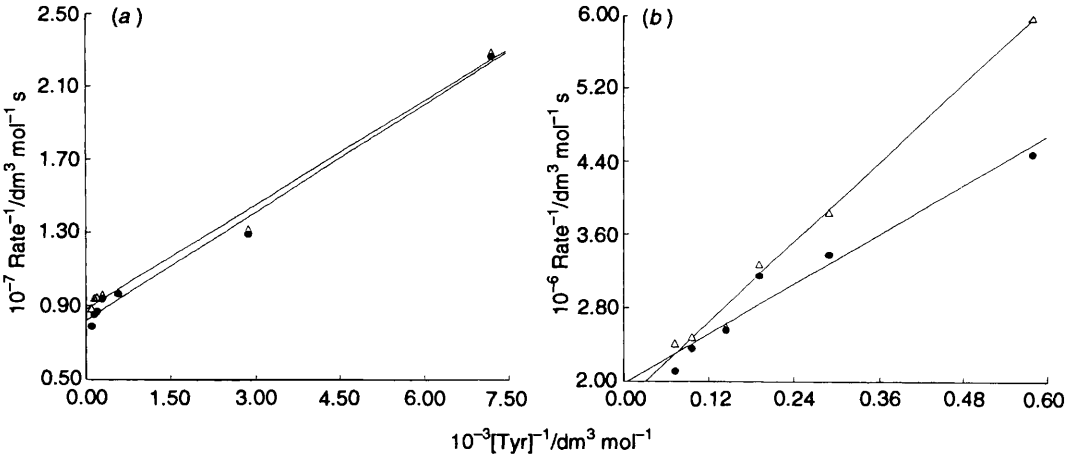


Fig. 6 Plots of 1/rate *vs.* 1/[substrate] (Lineweaver–Burk plots) for the oxidation of L- (△) and D-tyrosine methyl ester (●) catalysed by the deuterohaemin–peptide complexes **3** (*a*) and **4** (*b*) in CH_2Cl_2–tfe (9:1) in the presence of *tert*-butyl hydroperoxide.

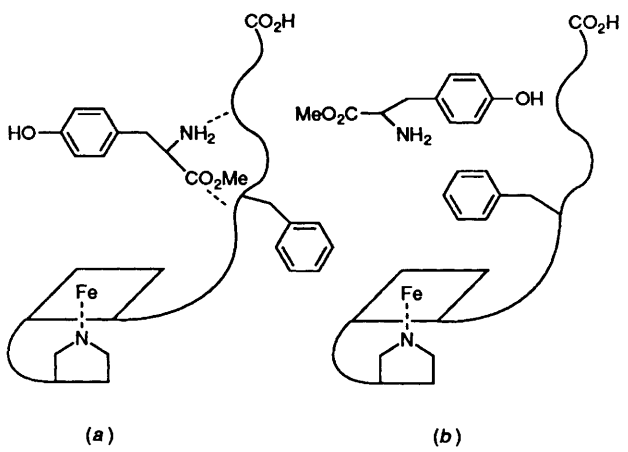


Fig. 7 Proposed models for catalyst–substrate interaction in the oxidation of L- and D-tyrosine by **4** (*a*) and **3** (*b*)

is typical of substrate inhibition.[17] This is shown in Fig. 5 for the oxidation of L-tyrosine. Unfortunately, it was not possible to extract accurate parameters for the inhibition effect by analysis of the kinetic data because the limited solubility of the substrates prevented extension of the investigation to the significant concentration range above the presumed K_m (in the millimolar range).[7] The origin of the inhibition, however, is the binding of the tyrosinate anion to iron(III) in the alkaline medium as shown by independent binding experiments with a large excess of ligand (λ_{max} 402 nm for the adduct). In this respect complexes **3** and **4** exhibit much higher tendency to bind the phenolate substrate with respect to **1** and **2**, because the presence of the covalently bound axial imidazole enhances the affinity for the sixth ligand. Inhibition by substrate co-ordination was in fact not observed in the oxidation catalysed by **1** and **2** under the same conditions.[7]

Since it was impossible to follow the model peroxidase reactions in neutral aqueous medium, where deprotonation of the phenolic substrates is negligible, because of the insolubility of the deuterohaemin–peptide complexes **3** and **4**, we thought to investigate them in an organic solvent [tfe–CH_2Cl_2 (1:9, v/v)], using the methyl esters of L- and D-tyrosine as substrates and *tert*-butyl hydroperoxide as oxidizing agent. Under these conditions no substrate inhibition was observed. The initial rates of the oxidations were found to increase linearly with the substrate concentration in the low concentration range, and to approach limiting values at high substrate concentrations. This behaviour suggests that the same kinetic scheme involving the formation of an intermediate complex between substrate and catalyst-active species and the subsequent irreversible

Table 3 Kinetic parameters for the catalytic oxidation of L- and D-tyrosine methyl ester by *tert*-butyl hydroperoxide in tfe–CH_2Cl_2 (1:9) at 20 °C

Catalyst	Substrate	k_{cat}/s^{-1}	K_m/mmol dm^{-3}	$k_{cat}K_m^{-1}$/ dm^3 mol^{-1} s^{-1}
3	L-Tyr–OMe	0.16	0.23	0.71
3	D-Tyr–OMe	0.17	0.24	0.70
4	L-Tyr–OMe	0.50	4.47	0.11
4	D-Tyr–OMe	0.45	2.53	0.18

decomposition of this complex in a rate-determining step, which characterized the behaviour of **1** and **2**,[7] can be applied here. Using the standard Michaelis–Menten relationship describing conventional enzymatic catalysis,[18] the parameters K_m and V_{max} contained in the kinetic equation can be obtained from a double reciprocal plot of the rate *vs.* substrate concentration [equation (1)].

$$1/\text{rate} = 1/V_{max} + K_m/V_{max}[\text{substrate}] \quad (1)$$

The plots corresponding to the reactions catalysed by **3** and **4** are shown in Fig. 6 and the kinetic results are reported in Table 3 in terms of k_{cat}, the turnover per second, representing the maximum activity of the catalyst, K_m, the Michaelis constant, and the ratio k_{cat}/K_m, representing the activity of the catalyst at low substrate concentration. The last parameter is the most suitable to describe the stereoselectivity effects in the reaction because it emphasizes the interaction between catalyst and substrate.[19] As shown by the data in Table 3 there is a marked difference in the chiral discrimination ability of the two deuterohaemin complexes towards the L and D forms of the substrate. Since any stereoselective effect must arise from some interaction between the substrate and the chiral peptide backbone it is clear that this interaction is different for **3** and **4**. This is shown by the K_m values, which are significantly different for the L and D isomers in the case of **4**, but are practically identical for **3**. This result is interesting because the complexes **3** and **4** differ solely in the position of the L-phenylalanine residue along the poly-L-alanine peptide chain. Therefore, we believe that chiral recognition of the substrate by the peptide chain of **4** occurs through interactions with the polar groups (amino and carboxymethyl) adjacent to the α-carbon atom, as shown schematically in Fig. 7(*a*), while in the case of **3** the hydrophobic interaction between the phenyl nucleus of the Phe residue and the phenol group of the substrate, which is far from the α-carbon atom, is probably preferred [Fig. 7(*b*)]. This result is not unexpected if we think that this hydrophobic interaction may

be enhanced by the aromatic ring stacking interaction with the porphyrin ring in which the phenyl nucleus of the Phe residue in **3** is apparently engaged. In conclusion, although the stereoselectivity effects observed in the model peroxidase reaction are moderate, the present deuterohaemin–peptide complexes offer an opportunity to show the appearance of chiral discrimination in a model peroxidase reaction. Similar mechanisms are operative in the enzymatic oxidations of the tyrosines and other substituted phenols,[6] where, of course, the possibility of multiple interactions between the peptide chain and the substrates and the effective immobilization of the substrate can, in some cases, dramatically increase the chiral discrimination.[14]

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References

1 *The Porphyrins*, ed. D. Dolphin, Academic Press, New York, 1978.
2 H. B. Dunford and J. S. Stillman, *Coord. Chem. Rev.*, 1976, **19**, 187; T. L. Poulos, *Adv. Inorg. Biochem.*, 1988, **7**, 1; J. H. Dawson, *Science*, 1988, **240**, 433; J. E. Frew and P. Jones, *Adv. Inorg. Bioinorg. Mech.*, 1984, **3**, 176.
3 P. R. Ortiz de Montellano, *Acc. Chem. Res.*, 1987, **20**, 289; *Cytochrome P-450: Structure, Mechanism & Biochemistry*, ed. P. R. Ortiz de Montellano, Plenum, New York, 1986.
4 F. P. Guengerich, *J. Biol. Chem.*, 1991, **266**, 10019; T. D. Porter and M. J. Coon, *J. Biol. Chem.*, 1991, **266**, 13469.
5 W. M. Atkins and S. G. Sligar, *Biochemistry*, 1988, **27**, 1610; *J. Biol. Chem.*, 1988, **263**, 18842; *J. Am. Chem. Soc.*, 1989, **111**, 2715; R. Raag and T. L. Poulos, *Biochemistry*, 1991, **30**, 2674; T. Shimizu, A. J. M. Sadeque, G. N. Sadeque, M. Hatano and Y. Fujii-Kuriyama, *Biochemistry*, 1991, **30**, 1490; B. A. Swanson, D. R. Dutton, J. M. Lunetta, C. S. Yang and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 1991, **266**, 19258; P. R. Ortiz de Montellano, J. A. Fruetel, J. R. Collins, D. L. Camper and G. H. Loew, *J. Am. Chem. Soc.*, 1991, **113**, 3195.
6 J. Sakurada, S. Takahashi and T. Hosoya, *J. Biol. Chem.*, 1986, **261**, 9657; S. Modi, D. V. Behere and S. Mitra, *Biochim. Biophys. Acta*, 1989, **996**, 214; L. Casella, M. Gullotti, S. Poli, M. Bonfà, R. P. Ferrari and A. Marchesini, *Biochem. J.*, 1991, **279**, 245; T. Hosoya, J. Sakurada, C. Kurokawa, R. Toyoda and S. Nakamura, *Biochemistry*, 1989, **28**, 2639; L. Casella, M. Gullotti, R. Ghezzi, S. Poli, T. Beringhelli, S. Colonna and G. Carrea, *Biochemistry*, 1992, **31**, 9451; T. Shimokawa and W. L. Smith, *J. Biol. Chem.*, 1991, **266**, 6168.
7 (*a*) L. Casella, M. Gullotti, L. De Gioia, E. Monzani and F. Chillemi, *J. Chem. Soc., Dalton Trans.*, 1991, 2945; (*b*) L. Casella, M. Gullotti, E. Monzani, L. De Gioia and F. Chillemi, *Rend. Fis. Acc. Lincei*, 1991, **9**, 2, 201.
8 G. S. Bayse, A. W. Michaels and M. Morrison, *Biochim. Biophys. Acta*, 1972, **284**, 34; S. Ohtaki, N. Nakagawa, M. Nakamura and Y. Yamazaki, *J. Biol. Chem.*, 1982, **257**, 761, 13398; X. Zheng and R. B. van Huystee, *Plant Cell, Tissue Organ Cult.*, 1991, **25**, 35; A. M. Lambeir, H. B. Dunford, R. B. van Huystee and J. Lobarzewski, *Can. J. Biochem. Cell Biol.*, 1985, **63**, 1086.
9 T. G. Traylor, *Acc. Chem. Res.*, 1981, **14**, 102; D. Mansuy, P. Battioni and J.-P. Battioni, *Eur. J. Biochem.*, 1989, **184**, 267; D. Mansuy, *Pure Appl. Chem.*, 1990, **62**, 741; M. J. Gunter and P. Turner, *Coord. Chem. Rev.*, 1991, **108**, 115.
10 J. H. Fuhrhop and K. M. Smith, in *Laboratory Methods in Porphyrin and Metalloporphyrin Research*, Elsevier, Amsterdam, 1975, p. 17.
11 E. Atherton, C. J. Logan and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 538.
12 (*a*) M. Momenteau, M. Rougee and B. Loock, *Eur. J. Biochem.*, 1976, **71**, 63; (*b*) M. Okuyama, T. Murakami, T. Nozawa and M. Hatano, *Chem. Lett.*, 1982, 111.
13 D. Seebach, A. Thaler and A. K. Beck, *Helv. Chim. Acta*, 1989, **72**, 857.
14 L. Casella, M. Gullotti, C. Selvaggini, S. Poli, T. Beringhelli and A. Marchesini, submitted for publication.
15 H. M. Goff, in *Iron Porphyrins, Part I*, eds. A. B. P. Lever and H. B. Gray, Addison-Wesley, Reading, MA, 1983, p. 237; I. Bertini and C. Luchinat, *NMR of Paramagnetic Molecules*, Benjamin/Cummings, Menlo, CA, 1986, ch. 7.
16 Y.-P. Huang and R. J. Kassner, *J. Am. Chem. Soc.*, 1981, **103**, 4927; O. K. Medhi and J. Silver, *Inorg. Chim. Acta*, 1988, **153**, 133.
17 T. Palmer, in *Understanding Enzymes*, Ellis Horwood, Chichester, 1985, ch. 8.
18 A. Fersht, *Enzyme Structure and Mechanism*, 2nd edn., Freeman, New York, 1985, ch. 3.
19 C. S. Chen and C. J. Sih, *Angew. Chem., Int. Ed. Engl.*, 1989, **28**, 695.

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