Axial Imidazole Distortion Effects on the Catalytic and Binding Properties of Chelated Deuterohemin Complexes

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Received February 9, 1995[⊗]

The effect of strain in the axial coordination of imidazole to the heme has been studied in the chelate complexes deuterohemin−histidine (DH-His) and deuterohemin−alanylhistidine (DH-AlaHis). Molecular mechanics calculations indicate that three types of distortion of the axial ligand occur in DH-His, due to the relatively short length of the arm carrying the donor group: tilting off-axis, tipping, and inclination of the imidazole plane with respect to the axial Fe−N bond. The effects of tilting ($\Delta\gamma \sim 10°$) and inclination of the imidazole ring ($\Delta\delta \sim 17°$) are dominant, while tipping is small and is probably of little importance here. By contrast, the axial imidazole coordination is normal in DH-AlaHis and other computed deuterohemin−dipeptide or −tripeptide complexes where histidine is the terminal residue, the only exception being DH-ProHis, where the rigidity of the proline ring reduces the flexibility of the chelating arm. The distortion in the axial iron−imidazole bond in DH-His has profound and negative influence on the binding and catalytic properties of this complex compared to DH-AlaHis. The former complex binds more weakly carbon monoxide, in its reduced form, and imidazole, in its oxidized form, than the latter. The catalytic efficiency in peroxidative oxidations is also reduced in DH-His with respect to DH-AlaHis. The activity of the latter complex is similar to that of microperoxidase-11, the peptide fragment incorporating the heme that results from hydrolytic cleavage of cytochrome *c*.

Introduction

A key feature in the active-site structure of heme proteins is the presence of a basic amino acid side chain axially ligated to the iron center of the cofactor, on the side opposite to which the catalytic reaction occurs.[1] Thus, in peroxidases the nitrogen donor of a histidine imidazole is the iron axial ligand, whereas in cytochrome P-450 monooxygenases and chloroperoxidase this ligand is a cysteine thiolate and in catalases it is a tyrosine phenolate. It is generally agreed that the nature of the ligand axial to the heme iron is an important factor in the control of the reactivity of the enzyme.[2] This has been shown in several model studies on the catalytic[3] and electronic[4] properties of iron and manganese porphyrin complexes. Proteins can modulate the contribution of the axial ligand through more subtle effects,

though. One example is the hydrogen-bonding interaction between a carboxylate group and the proximal histidine in peroxidases,[5] which confers imidazolate character on the iron ligand and whose effects have been reproduced to some extent in model heme complexes.[6] Another example is the control of the axial (imidazole) ligand plane in the six-coordinate hemes of cytochromes *b* and *c*,[7] which is also studied in model iron porphyrin complexes.[8] A further possibility is the axial ligand distortion from the perpendicular to the heme plane that could be controlled by appropriate steric interaction or conformational strain in the proximal region. The possible role of this distortion, resulting from tilting of the proximal imidazole, in the control

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.

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of heme proteins activity has been recognized[9] and is currently considered an important factor determining the low dioxygen affinity of T-state hemoglobin.[10] The transition from R-state to T-state is mimicked to some extent by the picket fence hemes Fe(TpivPP)(1-MeIm)O$_2$ and Fe(TpivPP)(2-MeIm)O$_2$,[11] where strain in the proximal imidazole bonding is introduced by the steric demand of the 2-substituent.

In synthetic porphyrin complexes such an axial distortion can be easily introduced through chelating arms, bearing the donor groups, which are of relatively short length.[12] The effect of strain in the axial iron−ligand interaction of these chelated heme complexes has been apparently studied only in the case of heme−CO dissociation rates.[13] In this paper we report an investigation of the effect of axial ligand distortion on the binding and catalytic properties of chelated deuterohemins by examining the behavior of the complexes DH-His, and DH-AlaHis, where the axial ligand is the imidazole group of a
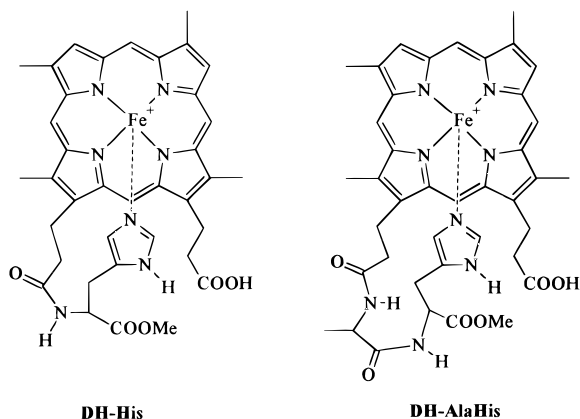
DH-His **DH-AlaHis**

chelated histidine residue. The conformational constraints of the chelating arms of DH-His, DH-AlaHis, and other related model complexes have been investigated by molecular mechanics calculations.

Experimental Section

Materials and Instrumentation. Compounds accessible from commercial sources were of the highest grade available. Deuterohemin was prepared from hemin[14] and microperoxidase-11 (MP-11) from cytochrome *c*[15] according to published methods. Deuterohemin−2(18)-L-histidine methyl ester (DH-His) was obtained as described in our previous study.[16] Dimethylformamide was purified by treatment with barium oxide and distilled from calcium hydride under reduced pressure. Samples of the Fe(II)−CN$^-$ derivatives of the complexes for NMR studies were prepared under an inert atmosphere by reducing the Fe-(III)−CN$^-$ species in DMSO-d_6 with a degassed sodium dithionite solution in deuteriated water. Samples of reduced deuterohemins for optical studies were prepared in degassed MeOH−H$_2$O (3:1, v/v) solutions, under argon, using cells fitted with Schlenk connections, by adding small amounts of concentrated aqueous sodium dithionite solution to the hemin solution. The carbon monoxide adducts of the deuteroheme complexes were obtained by exposing the reduced solutions to carbon monoxide at atmospheric pressure.

Optical absorption spectra were recorded with an HP-8452A diode-array spectrophotometer, and NMR spectra, on a Bruker AC200 spectrometer operating at 200 MHz. FABMS spectra were obtained with a VG7070 EQ spectrometer and CD spectra were recorded on a Jasco J 710 dichrograph using quartz cells of 0.1−10 cm path length.

Preparation of Alanyl-L-histidine Methyl Ester. Z-L-Ala-OH (Z = N-(benzyloxy)carbonyl, 89.6 mmol) and pentafluorophenol (98.5 mmol) were dissolved in ethyl acetate (200 mL). A solution of dicyclohexylcarbodiimide (DCC) (89.6 mmol) in ethyl acetate was then added under stirring at 5 °C, and the mixture was allowed to react overnight under stirring. The dicyclohexylurea thus formed was filtered off and washed with ethyl acetate. The filtrate was evaporated to dryness under vacuum, and the crude Z-L-Ala-PFP ester was crystallized by dissolution in the minimum amount of ethyl acetate and precipitation with petroleum ether (yield 82%).

L-Histidine methyl ester (16.6 mmol) and 1 equiv of triethylamine were dissolved in anhydrous DMF. The Z-L-Ala-PFP ester (16.6 mmol) was then added, and the mixture was allowed to react overnight with stirring at room temperature. The reaction was followed by TLC on silica gel using a mixture of chloroform, acetic acid, and hexane (8:1:1, v/v/v/) as eluent. The reaction mixture was filtered, and the filtrate was evaporated to dryness under vacuum. The residue was dissolved in ethyl acetate and washed twice with water in a separatory funnel. The organic phase was dried (sodium sulfate) and evaporated to dryness. The residue was treated with petroleum ether and filtered off. The crude product was dissolved in the minimum amount of ethanol−ethyl acetate (1:2, v/v) and then precipitated with petroleum ether. The Z-L-Ala-L-HisOMe product was collected by filtration and dried under vacuum (yield 70%). This showed a single TLC spot (silica gel, ethanol−water, 1:1, v/v) with $R_f = 0.8$.

Anal. Calcd for C$_{18}$H$_{22}$N$_4$O$_5$: C, 57.75; H, 5.92; N, 14.96. Found: C, 57.70; H, 6.13; N, 15.05. ^{1}H NMR (DMSO-d_6): δ 1.17 (d, 3 H, CH$_3$−C), 2.89 (d, 2 H, CH$_2$−imidazole), 3.56 (s, 3 H, OCH$_3$), 4.05 (t, 1 H, His α-CH), 4.40 (q, 1 H, Ala α-CH), 5.00 (s, 2 H, CH$_2$−Ph), 6.83 (s, 1 H, imidazole 5-H), 7.2−7.4 (~s, 5 H, Ph H), 7.59 (s, 1 H, imidazole 2-H), 8.1,8.2, 8.3 (~s, 3 H, NH). MS, *m/z* (%): 117 (62), 136 (84), 137 (24), 155 (27), 165 (1), 184 (100), 185 (14), 374 (M$^+$, 2).

Z-L-Ala-L-HisOMe (13.4 mmol) was dissolved in the minimum amount of methanol−acetic acid (3:1, v/v), and then, under nitrogen, palladium−charcoal (10% palladium content) (1 g) was added. The mixture was cooled to 10 °C and 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 oil thus obtained was treated several times with diethyl ether, filtered off, washed again with diethyl ether, and dried under vacuum over potassium hydroxide (yield 95%). The L-Ala-L-HisOMe product showed a single TLC spot (silica gel, 1-butanol−acetic acid−water, 4:1:1, v/v/v) with $R_f = 0.2$.

Anal. Calcd for C$_{10}$H$_{16}$N$_4$O$_3$: C, 49.99; H, 6.71; N, 23.32. Found: C, 50.41; H, 6.31; N, 23.89. ^{1}H NMR (DMSO-d_6): δ 0.98 (d, 3 H, CH$_3$−C), 2.8−3.0 (m, AB part of an ABX system, 2 H, CH$_2$−imidazole), 3.4 (br, 2 H, NH$_2$), 3.83 (q, 1 H, Ala α-CH), 4.05 (dt, 1 H, His α-CH), 6.81 (s, 1 H, imidazole 5-H), 7.55 (s, 1 H, imidazole 2-H), 7.93 and 8.05 (~s, 2 H, NH). MS, *m/z* (%): 81 (68), 82 (100), 95 (25), 110 (25), 154 (42), 196 (4), 197 (9), 208 (53), 240 (M$^+$, 7).

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Preparation of Deuterohemin−2(18)-L-Alanyl-L-histidine Methyl Ester (DH-AlaHis). This compound was prepared by following a procedure similar to that employed for the synthesis of DH-His.[16] To a solution of deuterohemin chloride (1 mmol) in anhydrous DMF were added 1-hydroxybenzotriazole (3 mmol) and, after 0.5 h, dicyclohexylcarbodiimide (1 mmol) under stirring at 0 °C. The mixture was kept under stirring at 0 °C for 1 h. Then, L-Ala-L-HisOMe (1 mmol) dissolved in dry DMF containing a small amount of anhydrous lithium chloride (to allow complete dissolution)[17] was added. The reaction was continued at 0 °C for 4 h, followed by 6 d at room temperature. The progress of the reaction was monitored by TLC on silica gel using a mixture of *1*-butanol−acetic acid−water (4:1:1, v/v/v) as eluent. The reaction mixture was taken to dryness under vacuum, and the residue was treated several times with diethyl ether and water, in order to eliminate the unreacted peptide and lithium chloride. The crude 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 first fraction consists of unreacted deuterohemin, which is followed by the desired product. This was recovered upon evaporation to dryness of the solution under vacuum (yield 30%). The product 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 was characterized by UV and NMR spectroscopy and by FABMS.

Ligand Binding. The equilibrium constants for the binding of imidazole to DH-His and DH-AlaHis were determined by spectrophotometric titration at 25 °C. The data were analyzed as described previously.[18]

Kinetics. Kinetic experiments on the catalytic oxidation of *N*-acetyl-L-tyrosine by hydrogen peroxide in the presence of hemin catalysts were carried out as follows. The reaction mixture contained 2.4 × 10^{-8} M deuterohemin complex, 8.4 × 10^{-4} M hydrogen peroxide, and 2.4 × 10^{-4}−4.9 × 10^{-3} M *N*-acetyl-L-tyrosine in 0.01 M borate buffer at pH 9.0. The kinetic studies were performed in thermostated cells equipped with a magnetic stirrer at 20 ± 0.1 °C. The reactions were initiated by the addition of the oxidant and followed by the growth of the absorption band at 316 nm due to the formation of the dimerization products. Initial rates were calculated using the difference in molar absorption coefficients between the oxidation products and tyrosine at 316 nm at pH 9.0, $\Delta\epsilon = 4600$ M^{-1} cm^{-1}. This value was determined by enzymatic oxidation of *N*-acetyl-L-tyrosine by horseradish peroxidase and hydrogen peroxide, as recently described.[19] Hemin destruction was negligible within the time needed for rate determination.

Molecular Mechanics. Molecular modeling experiments were performed using the Biosym programs Insight and Discover.[20,21] A modified version of the CVFF[22] force field derived to study iron porphyrin molecules[23] was used in molecular mechanics (MM) and molecular dynamics (MD) calculations. For each complex a preliminary MM geometry optimization was carried out and the resulting minimum energy conformer was used as a starting conformation for a short MD run (5000 steps of one fs at 300 K) which was expected to produce a quite well-equilibrated molecular system. To search the molecular energy hypersurface, conformations obtained every 100 steps of MD were energy-minimized using the conjugate gradient algorithm. The minimized structure featuring the lowest energy value was considered as the most representative for that molecule. One of the main goals of our MM and MD study was the evaluation of energetics involved in the formation of the pentacoordinated species, where the axial ligand is represented by the histidine group at the end of the substituent arm. Both Nδ1 and Nε2 were studied as possible coordinating atoms of histidine. For all the studied complexes the MM energy was largely favorable for the Nε2 coordination (data not shown).

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Table 1. Energies for the Four- to Five-coordination Transformation of the Hemin Complexes[a]

complex[b]	ΔE[c]	complex[b]	ΔE[c]
DH-His	−12.35	DH-β-AlaHis	−30.97
DH-ProHis	−19.88	DH-AlaHis	−29.14
DH-D-AlaHis	−30.77	chelated protohemin[d]	−31.01
DH-AlaAlaHis	−30.52		

[a] All energies in kcal mol^{-1} ($\approx$4.184 kJ mol^{-1}). [b] The absolute configuration of chiral amino acid residues is L unless otherwise stated. [c] ΔE represents the difference between the energies of the five- to four-coordinated forms. [d] Reference 24.

During a completely unconstrained simulation, a very marked tendency was observed for the imidazole ring to assume a stacked conformation in which the ring itself is nearly parallel to the macrocycle. However this conformation does not correspond to a well-defined N−Fe coordinative interaction. In order to compute a "reference" energy for a purely tetracoordinated form, we decided to carry out a constrained simulation in which the imidazole ring lies at a distance not smaller than 10 Å from the metal center. It must be noted, in addition, that all the computations reported here have been performed on "in vacuo" molecules, thus neglecting all solvent effects. These are, in general, quite important for determining preferred molecular conformations. In particular, hydroxide ion could play a role in the coordination chemistry of the porphyrin complexes at basic pH. However, a MM force field able to properly describe changes in coordination number has not yet been described. This made impossible a more precise evaluation of the energetics of the five-coordination reaction. For these reasons the MM energy trend has to be considered as semiquantitative.

Results

MM Calculations. The conformational properties of a series of homologous compounds (Table 1) were investigated using the procedure outlined above. All the molecular species are characterized by the presence of an amino acid or a short peptide linked to the carboxylic group of a deuterohemin propionic acid chain. Two different conformations were considered for each complex. The first one is characterized by lack of coordination of the nitrogens of the terminal histidine residue, while in the second conformation the peptide chain is folded and one of the histidine nitrogens is allowed to act as the fifth ligand. As is well-known, when working with the MM approach, one cannot compare absolute energy values for different molecules. On the contrary, only the relative conformational energy differences of the same molecule may have well-defined physical meaning. The computed energy differences which accompany the formation of pentacoordinated forms are assumed to be reliable because in the porphyrin complexes considered here the fifth ligand is covalently linked, being part of the porphyrin molecule itself. Moreover, the MM force field used in this investigation was explicitly developed to allow an accurate MM description of both four- and five-coordinate porphyrin species, using exactly the same set of force field parameters.[23]

Table 1 shows the energy difference obtained for the four- to five-coordination transitions. There is a clear tendency toward the five-coordinate species for all the complexes investigated. However, the energy gain is quite variable, suggesting that the length and composition of the chelating arm may strongly affect the stability of the five-coordinate species. In particular, the DH-His complex is characterized by the lowest energy gain, indicating that a chain containing a single amino acid residue does not allow a strong axial interaction by the imidazole group. All the deuterohemin complexes with two amino acid residues in the chelating arm are characterized by more favorable energy gains with respect to the DH-His complex. This shows that, independent of composition, a chelating arm with two residues has suitable characteristics to
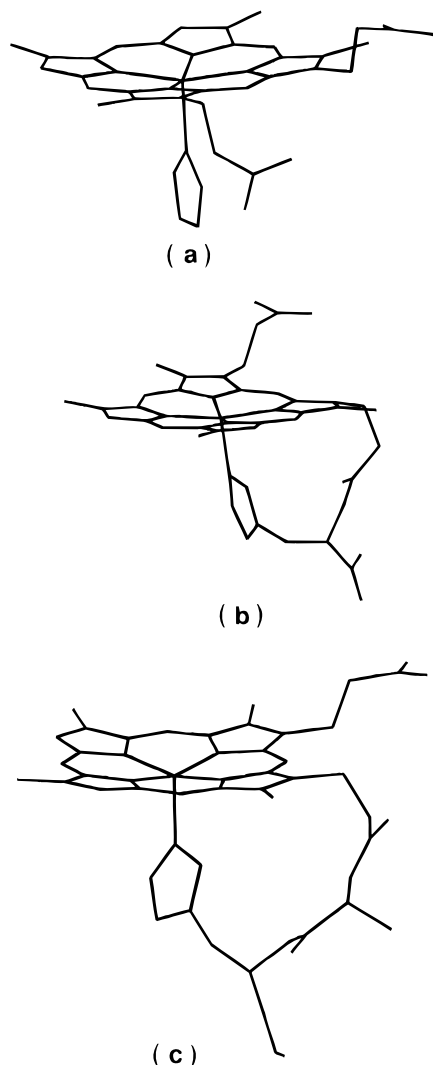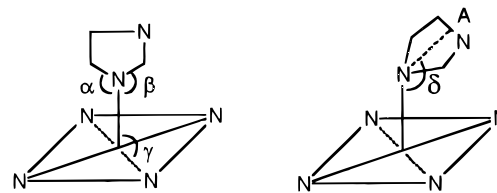
Figure 1. MM energy-minimized structures of:(a) the complex between deuterohemin and imidazole, (b) DH-His in five-coordinated form, and (c) DH-AlaHis in five-coordinated form. Hydrogen atoms are omitted for clarity.

Angle	DH-Imidazole	DH-His	DH-AlaHis
α	130.1	125.6	129.9
β	129.4	131.3	129.7
γ	97.9	88.5	97.8
δ	179.8	162.7	179.5

Figure 2. Definition of the angles characterizing the distortion of an imidazole axially bound to deuterohemin: tilting off-axis (γ), tipping ($\alpha \neq \beta$), inclination (δ = FeNA). The values reported (in degrees) refer to energy-minimized structures.

allow an unstrained coordination. A representation of the energy-minimized DH-His and DH-AlaHis structures is shown in Figure 1. Interestingly, DH-His, even if characterized by a more strained chelating arm than DH-AlaHis, does not show a more domed porphyrin ring. A close examination of the MM-derived structures permits an explanation of this observation. The strained chelating arm of DH-His has a tendency to pull the iron atom outside the porphyrin plane, favoring the doming of the macrocycle. However, the propionic acid chain linked to the coordinated histidine tends to dome and distort the ring in an opposite way, again due to the strain present in the molecule. The resulting effect on the macrocycle of DH-His is a twisting distortion of the porphyrin ring. The energy-minimized structure of the adduct between deuterohemin and an axially-bound imidazole group is also included as an appropriate reference. Interestingly, the complex with three amino acid residues in the chain shows a behavior similar to that of the deuterohemin−dipeptide complexes. In particular cases, the amino acid composition can have an influence on the coordination tendency; the chelating chain containing a proline residue gives only partial stabilization to the five-coordinate deuterohemin complex, a result explainable with the constrained conformational characteristics of the cyclic imino acid. It is noteworthy that all the energy values found for unstrained deuterohemin−peptide complexes are very close to

that obtained for a chelated protohemin complex designed by Traylor et al. to avoid chelating strain.[24]

The distortion of the axial imidazole in DH-His can be characterized by the four angles α, β, γ, δ defined in Figure 2. The optimum values of these angles were obtained from energy-minimized structures of a five-coordinate deuterohemin containing an axial imidazole group, or the DH-His and DH-AlaHis residues.

Characterization of DH-AlaHis. The deuterohemin complex DH-His has been characterized elsewhere.[14,25] The FABMS spectrum of DH-AlaHis, obtained from a 3-nitrobenzyl alcohol matrix, showed the molecular ion cluster centered at *m/z* 803, corresponding to the hydroxide adduct of the complex. Computer simulation of the molecular ion cluster using the natural abundance of isotopes gave excellent agreement with the experimental pattern. The isomeric composition of DH-AlaHis, containing the peptide substitution at positions 2 and 18 of the porphyrin ring, and the occurrence of molecular association in the concentration range of the solutions required to record the NMR spectra make impossible a detailed interpretation of these spectra, as for DH-His and other chelated deuterohemin complexes prepared by covalent linkage to one of the porphyrin carbonyl groups.[16,18,26] The presence of an alanyl residue in DH-AlaHis makes the proton NMR spectrum of the diamagnetic Fe(II)−CN⁻ species richer in the aliphatic region compared with the corresponding spectrum of DH-His,[16] with a prominent methyl signal near δ 1.2. In the paramagnetic DMSO-d_6 spectrum of the high-spin Fe(III) species, the pattern of several broad signals occurring between 50 and 70 ppm, due to the porphyrin methyl and α-methylene groups, indicates a six-coordinate species in this solvent.[27] The low solubility of DH-AlaHis prevents the investigation in less polar solvents.

Spectra and Binding Experiments. In methanol or aqueous methanol, the electronic spectra of DH-His and DH-AlaHis are rather similar to that of deuterohemin, indicating that binding of an axial imidazole has a minor effect on the spectrum, as observed for protohemin complexes.[28] Equilibria with base-dissociated species for the strained DH-His complex were

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Table 2. Electronic Spectral Data for Deuterohemin Complexes

| complex | species | solvent | λ_{max} (nm) | | | |
			Soret	β	α	α/β
DH[a]	Fe(III)	MeOH−H$_2$O	388	490	612	
	Fe(II)	MeOH−H$_2$O	408	532	550	
	Fe(II)−CO	MeOH−H$_2$O	398	520	554	1.01
DH-His	Fe(III)	MeOH−H$_2$O	390	492	616	
	Fe(II)	MeOH−H$_2$O	414		542	
	Fe(II)−CO	MeOH−H$_2$O	408	526	554	0.82
DH-AlaHis	Fe(III)	MeOH−H$_2$O	390	492	620	
	Fe(II)	MeOH−H$_2$O	420		546	
	Fe(II)−CO	MeOH−H$_2$O	408	528	620	0.75
DH-Mb[b]	Fe(III)	aqueous buffer	393	496	620	
	Fe(II)	aqueous buffer	421		544	
	Fe(II)−CO	aqueous buffer	409	528	554	0.64
DH-Hb[c]	Fe(III)	aqueous buffer	394	500	620	
	Fe(II)	aqueous buffer	421		544	
	Fe(II)−CO	aqueous buffer	409	528	556	0.76

[a] DH = deuterohemin. [b] DH-Mb = deuteromyoglobin. Data from ref 30. [c] DH-Hb = deuterohemoglobin. Data from ref 31.

excluded in methanol solution on the basis of previous CD studies.[29] In order to assess the eventual displacement of the strained axial imidazole by hydroxide, which is present in nonnegligible concentration in the aqueous buffer at pH 9.0 used for the binding and catalytic studies described below, we performed concentration-dependent CD studies at various pHs. In alkaline aqueous buffer, where the complexes are slightly soluble, porphyrin aggregation becomes significant at concentrations of about 1 μM, but deviations from Beer's law are negligible below 0.3 μM. As expected, in dilute solutions the CD spectrum of DH-His[16] is concentration independent at constant pH, since dilution does not affect the intramolecular equilibrium between imidazole-bound and -unbound forms. The CD spectra are also completely pH independent between pH 8.0 and 9.0, though the optical activity decreases by about 10% when the pH is raised to 9.3 and is reduced to less than 10% at pH 10.3, where it is likely that a hydroxide complex of the nonchelated hemin is mostly present in solution.

Distortion of the axial imidazole and, possibly, contributions by base-dissociated species may be responsible for the difference seen in the electronic spectra of the reduced DH-His and DH-AlaHis complexes, the latter of which is so similar to those of deoxydeuteromyoglobin[30] and deoxydeuterohemoglobin[31] (Table 2) that stable binding by the chelated imidazole seems warranted. Binding of the chelating arm is clearly enforced by coordination of exogenous ligands to the iron sixth position. This is shown by the spectral features of the Fe(II)−CO adducts of DH-His and DH-AlaHis, which are both similar to those of carbonyl deuteromyoglobin or carbonyl deuterohemoglobin and markedly different from those of the Fe(II)−CO adduct of deuterohemin (Table 2). However, the difference in strength of the axial imidazole−iron interactions in the CO adducts of DH-His and DH-AlaHis is indicated by the ratio of intensity of the visible α and β bands.[32] This ratio is lower, implying stronger Fe−imidazole interaction, for DH-AlaHis than for DH-His. Interestingly, the α/β ratio for the DH-AlaHis complex is the same as that for hemoglobin.

The different strengths of the axial Fe−imidazole interactions in DH-His and DH-AlaHis should be reflected in different
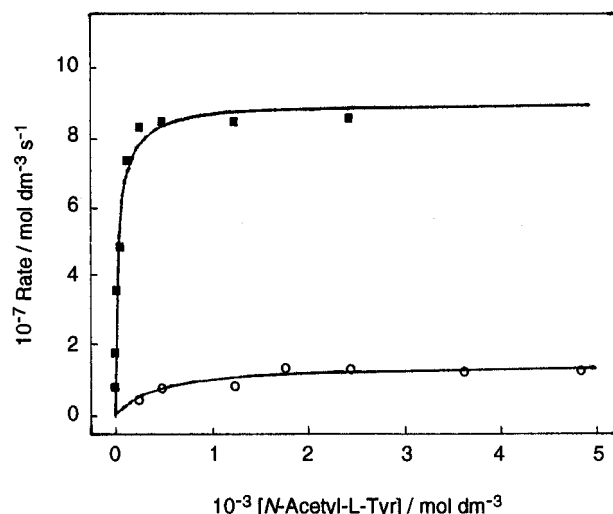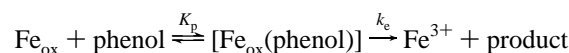
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Figure 3. Plots of rate *vs* substrate concentration for the oxidation of *N*-acetyl-L-tyrosine by hydrogen peroxide catalyzed by DH-His (○) and DH-AlaHis (■) in 0.01 M borate buffer, pH 9.0, at 20 °C.

Table 3. Binding Constants and Hill Coefficients Obtained by Spectral Titration of Chelated Deuterohemin Complexes with Imidazole

| complex | borate buffer, pH 9.0 | | CH$_2$Cl$_2$−CF$_3$CH$_2$OH, 9:1 (v/v) | |
	K (M^{-1})	n	K (M^{-1})	n
DH-His	150	1.02	$>10^6$	1.00
DH-AlaHis	580	0.95	$>10^7$	1.02

affinities of these complexes for an exogenous base like imidazole. For this ligand we determined the binding constants by spectrophotometric titrations, using dilute solutions of the deuterohemin complexes in aqueous borate buffer, pH 9.0, and in the less polar mixture CH$_2$Cl$_2$−CF$_3$CH$_2$OH, 9:1 (v/v). Clean isosbestic behavior was observed in all cases, the final spectra being typical of low-spin, six-coordinate bis(imidazole)deuterohemin complexes (Soret $\lambda_{max} \approx 400$ nm, visible $\lambda_{max} \approx 540$ nm); the stoichiometry of binding was 1:1. The binding constants (K) are reported in Table 3. As already observed,[16] the range of K values decreases strongly with the solvent polarity, but it is clear from these data that for the strained complex DH-His the affinity for the sixth axial ligand is markedly reduced.

Catalytic Oxidations. The effect of strain of the axial ligand on the catalytic activity of chelated deuterohemin complexes was studied by the kinetics of peroxidative oxidation of *N*-acetyl-L-tyrosine by hydrogen peroxide in aqueous buffer at pH 9.0. This reaction produces in the initial stages a mixture of dimeric phenol-coupling products that can be detected by the characteristic absorptions.[19] The kinetic data were obtained by the initial rate method to ensure that no catalyst destruction occurred during the measurements. Substrate saturation behavior was observed for both DH-His and DH-AlaHis catalysts (Figure 3), consistent with the concept that the reaction proceeds through the formation of a precursor complex:

$$\text{Fe}_{ox} + \text{phenol} \underset{}{\overset{K_p}{\rightleftharpoons}} [\text{Fe}_{ox}(\text{phenol})] \overset{k_e}{\longrightarrow} \text{Fe}^{3+} + \text{product}$$

In the peroxidase-catalyzed reactions the rate-determining step is usually the reaction of the phenol with the intermediate compound **II**, producing a phenoxy radical and the resting enzyme.[19,33] If a similar scheme is assumed here, the intermediate species Fe$_{ox}$ can be identified as an (Fe=O)$^{2+}$ species. In

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Table 4. Kinetic Parameters for the Catalytic Oxidation of
N-Acetyl-L-tyrosine by Hydrogen Peroxide in the Presence of
Chelated Hemin Complexes in Borate Buffer, pH 9.0, at 20 °C

catalyst	k_e (s^{-1})	K_p (M)	k_e/K_p (M^{-1} s^{-1})
DH-His	2.5	2.2×10^{-4}	1.1×10^4
DH-AlaHis	21	2.9×10^{-5}	7.2×10^5
MP-11	16	1.6×10^{-5}	1.0×10^6

the limit of preequilibrium, K_p represents the dissociation constant of the precursor complex and k_e the rate constant for intramolecular electron transfer. The kinetic parameters were obtained from double reciprocal plots of the rate *vs* substrate concentration (Table 4). The catalytic activity of unmodified deuterohemin is extremely low under the same conditions. However, the kinetic analysis was extended to the heme–undecapeptide microperoxidase-11 (MP-11),[15,34] which retains the proximal His-18 residue of cytochrome *c* and for this reason can be assumed as a useful reference compound for heme–axial imidazole interaction. This compound was recently reported to be an effective catalyst for sulfide oxidation, amine *N*-demethylation, and even olefin epoxidation in the presence of hydrogen peroxide.[35] As shown by the data in Table 4, the maximum catalytic activity of DH-AlaHis is similar to that of MP-11 and 1 order of magnitude greater than that of DH-His.

Discussion

Axial binding of an imidazole group in strained chelated hemin complexes can occur with three types of distortion: tilting off-axis, tipping, and inclination of the ring plane with respect to the axial Fe–N bond (Figure 2). These types of distortion are characterized by the four angles α, β, γ, δ. The completely undistorted structure has the imidazole coordinated in a symmetrical fashion (see Figure 1a), with the ring plane perpendicular to the plane identified by the four N atoms of the porphyrin. The situation for DH-AlaHis and the other complexes containing axially bound histidine dipeptide or tripeptide residues, with the exception of ProHis, is very close to the geometry of Figure 1a, whereas all types of distortion occur in the case of DH-His. The angles α and β deviate slightly from the cases depicted in Figure 1a,c. Larger deviations have been computed for the angles γ (about 10°) and δ (about 17°). A value of γ larger than 90° is always an indication that the porphyrin macrocycle undergoes a "doming" distortion. In the case of DH-His γ assumes a value smaller than 90°. This, however, is essentially due to the tilting of the Fe–N bond, while the doming of the macrocycle is largely preserved, even if accompanied by an evident loss of axial geometry. The conclusion is that, in the case of the DH-His complex, several geometrical contributions are responsible for the overall distorted coordination around the Fe center.

It is interesting to note that while tilting[11] and tipping[36] of the axial imidazole are well-known phenomena observed in the adducts formed by simple iron porphyrin complexes when the exogenous imidazole contains bulky substituents in ring position 2, the inclination of the imidazole plane, with respect to the perpendicular to the porphyrin plane ($\delta \neq 180°$), can be only induced in a chelated iron porphyrin complex. Although this type of distortion is unlikely to be present in native heme proteins, it was recently shown to occur in the structure of the H93G mutant of sperm whale myoglobin, where the proximal ligand (His 93) has been replaced by an exogenous imidazole.[37]

The presence of strain in the axial ligand bonding to iron porphyrin complexes is expected to have strong influence on the reactivity of the metal center. Some recent theoretical calculations indicate that even the well-known bonding distortion and reduced affinity of carbonmonoxy globin hemoproteins may be due mostly to strain in the proximal imidazole bonding rather than to distal effects.[39] Chelated heme complexes are clearly advantageous in the study of this effect in catalytic reactions, where the use of nonchelated complexes requires the addition of an excess of exogenous ligand that may undergo competitive reactions with the substrate. Besides the well-known effects on the affinity of model heme complexes for dioxygen and carbon monoxide,[11] little attention has been paid to the influence of axial ligand strain on the reactivity of hemin complexes. Traylor et al. have shown that the presence of strain causes a change in the CO dissociation mechanism of chelated mesoheme complexes,[13] but the introduction of strain was not obvious in the catalytic oxidations by chelated protohemin complexes, probably due to different substitution patterns at the porphyrin periphery between the complexes studied.[28] As is shown here, the decreased bonding interaction between the axial imidazole and the iron(III) center in strained chelated hemin complexes depresses both the affinity for a sixth axial ligand and the catalytic activity of the complex in the peroxidative reaction. Both effects are probably due to an altered electron distribution in the strained complex, as suggested for the carbonmonoxy heme complexes.[39] The decreased electron density at the iron atom provided by the strained axial bond is further expected to lower the stability of the high-valent iron oxidation state in the catalytically active intermediate[38] and reduce the rate of the hydroperoxide O–O bond cleavage in the step generating this intermediate,[2] as has been proposed for peroxidases.[1]

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