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Phosphine Binding as a Structural Probe of the Chloroperoxidase Active Site: Spectroscopic Evidence for Endogenous Thiolate Ligation to the Heme Iron

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In order to identify the endogenous axial ligand to the active-site heme iron of chloroperoxidase, the binding of phosphine ligands has been examined with UV–visible absorption, magnetic circular dichroism (MCD), EPR, and CD spectroscopy. The unusual spectral properties of the ferric and ferrous chloroperoxidase–phosphine adducts have been compared with those of analogous complexes of cytochrome P-450 (P-450). Bis(hydroxymethyl)methylphosphine binds to both ferric and ferrous chloroperoxidase in a molar ratio of 1:1 and with dissociation constants on the order of 10^{-3}–10^{-4} M to form homogeneous low-spin complexes. The resulting ferric enzyme complex exhibits a hyperporphyrin (split Soret) UV–visible absorption spectrum with maxima at 376, 450, and 553 nm that is essentially identical with the spectrum observed upon phosphine binding to ferric P-450-CAM. In contrast, a single Soret peak at 427 nm appears upon phosphine binding trans to histidine in ferric myoglobin and horseradish peroxidase. Close spectral similarities are also seen between the phosphine adducts of ferrous chloroperoxidase and P-450-CAM with both displaying substantially red-shifted Soret peaks at 457.5 nm. MCD spectra of the phosphine complexes of the two enzymes closely resemble each other as well, especially in the ferric case. In addition, the EPR parameters of phosphine-bound ferric chloroperoxidase and P-450 are quite comparable. Since the ligation of cysteine thiolate to the heme iron of P-450 has been well established, the results reported herein with four different spectroscopic methods, together with previous comparative studies of both enzymes from our laboratories and heme model studies by others, provide additional strong support for endogenous thiolate ligation to the heme iron of chloroperoxidase.

Introduction

Although no free cysteine residues available for ligation to the heme iron of chloroperoxidase have been detected in chemical analyses of the enzyme,[2] extensive spectroscopic similarities between analogous derivatives of chloroperoxidase and P-450[3] have provided substantial support for the presence of an endogenous thiolate ligand to the heme iron of chloroperoxidase as is well established for P-450. In particular, close correspondence between chloroperoxidase and P-450 derivatives have been observed with UV–visible absorption,[4] MCD,[3,5] EPR,[6] Mössbauer,[7,8] and extended X-ray absorption fine structure[9] spectroscopy. Recently, we have presented further evidence for the ligation of an endogenous thiolate to the heme iron of ferric chloroperoxidase: a characteristic hyperporphyrin[10] (split Soret) UV–visible absorption spectrum has been generated upon exogenous thiol ligation to ferric chloroperoxidase.[11] Such a unique "split Soret" spectrum has been shown to be characteristic of a bis(thiolato)ferric heme iron (RS–ferric heme–SR) coordination structure in previous studies of biomimetic ligand binding to heme models[12–14] and P-450.[14–16] Phosphines bind to both ferric and ferrous P-450 as well as to ferric heme–thiolate model complexes and also produce hyperporphyrin spectra.[12,14,17,18] In the present study, we report that phosphine adducts of ferric and ferrous chloroperoxidase also exhibit unusually red-shifted Soret UV–visible absorption bands at around 450 nm. Overall, spectral features typical of hyperporphyrin complexes are seen in both the optical absorption and MCD spectra of phosphine-bound chloroperoxidase. The unique spectral properties of phosphine/thiolate mixed-ligand heme iron complexes[12,14,17,18] provide particularly stringent tests for the presence of a thiolate ligand. Duplication of those properties in the phosphine adducts of chloroperoxidase thus provides especially strong support for the existence of an endogenous thiolate ligand bound to the active-site heme iron of *both* ferric and ferrous chloroperoxidase.

Experimental Section

Enzymes. Chloroperoxidase, purified from *Caldariomyces fumago* grown on fructose[19] as described by Morris and Hager[20] and Palcic et al.[21] was highly pure (>85%) as judged by sodium dodecyl sulfate gel electrophoresis and had an R_z (A_{399}/A_{280}) value of 1.47 or greater at pH 6.0 and 4 °C. Cytochrome P-450-CAM from *Pseudomonas putida* grown on *d*-camphor was purified to electrophoretic homogeneity ($A_{391}/A_{280} > 1.5$) following slight modifications[22] of the original methods of Peterson and co-workers[23] and of Gunsalus and Wagner.[24]

Reagents. Bis(hydroxymethyl)methylphosphine and dimethylphenylphosphine were purchased from Alfa Products, Morton Thiokol Inc. All other chemicals were of reagent grade from Aldrich or Fisher and were used without further purification.

Titrations of Chloroperoxidase with Phosphines and Data Analysis. Essentially the same procedures previously used with P-450-CAM[16] were employed in the present work except that titrations were performed under nitrogen atmosphere with Thunberg-type cuvettes and gastight micro syringes, Stock solutions of phosphines (0.1–1 M) were prepared anaerobically in absolute ethanol.

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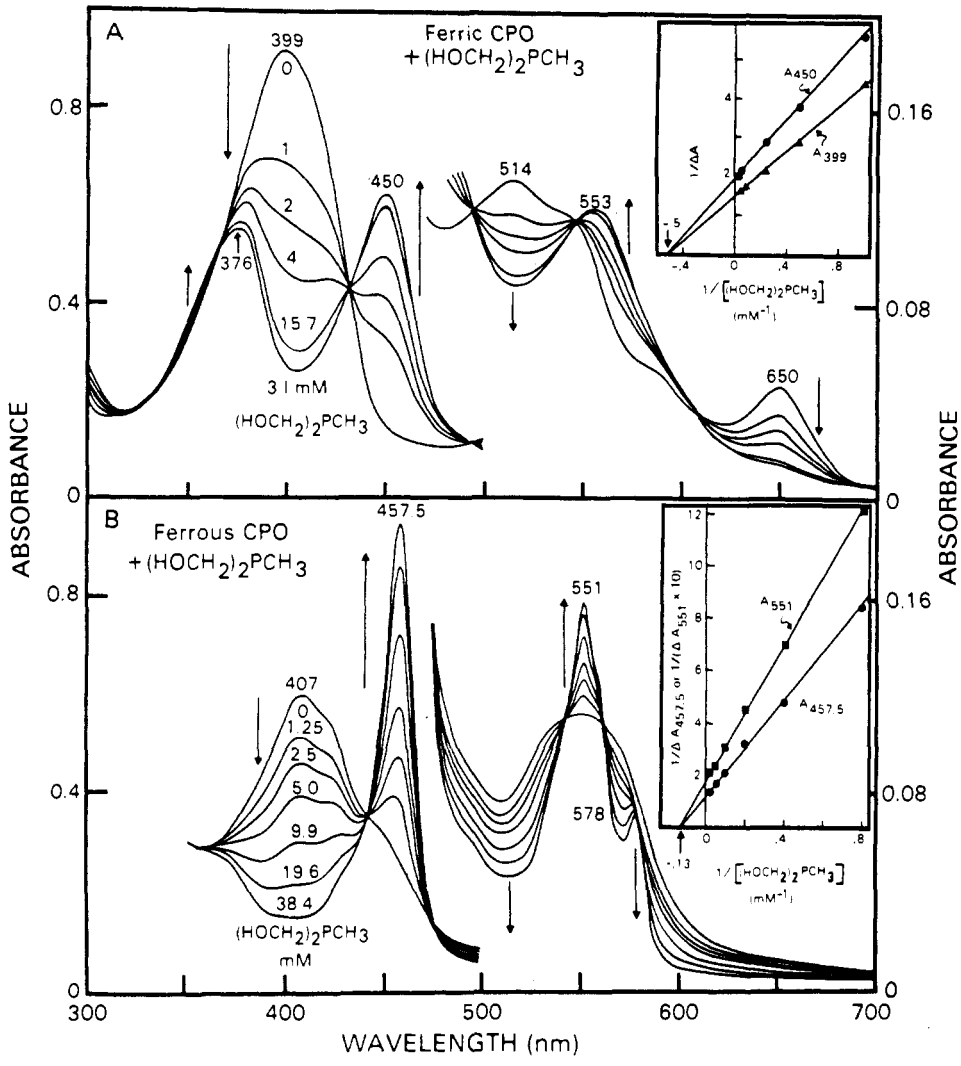

Figure 1. Spectrophotometric titrations of native ferric (A) and ferrous (B) chloroperoxidase (CPO) with bis(hydroxymethyl)methylphosphine. The titrations were performed with 9.4 μM (ferric) and 8.1 μM (ferrous) enzyme concentrations in 0.1 M potassium phosphate buffer, pH 6.0, and at 4 °C. The phosphine concentrations employed at each stage are indicated in each figure and the direction of the absorbance change is shown by arrows. Isosbestic points are seen at 364, 431.5, 494, 547, and 607 nm for the ferric enzyme and at 441, 471, 542, and 562 nm for the ferrous enzyme. For the ferrous enzyme, spectra in the region between 300 and 360 nm are omitted because of the strong absorbance of the dithionite used for reduction of the enzyme. The insets in the respective titrations show double-reciprocal plots of the results using the absorbance changes at the indicated wavelengths. See text for further details.

Spectrophotometric Measurements. UV–visible absorption spectra were recorded on Varian-Cary 219 or 210 spectrophotometers at 4 °C. MCD/CD spectra were measured at 4 °C on a Jasco J-40 spectropolarimeter with the electromagnetic field [14.7 kG (1.47 T)] direction parallel to the direction of light propagation. EPR spectra were obtained at 77 K on a Varian E-9 Century Series spectrometer equipped with a Varian E-102 microwave bridge. UV–visible absorption spectra were handdigitized in 2-nm wavelength intervals and inserted point by point onto an 8080A microprocessor-based computer system (Digital Specialities, Carrboro, NC). MCD/CD spectra were recorded in 1-nm wavelength intervals with the computer-based data acquisition and manipulation system. For further details, see Dawson et al.[22]

Results and Discussion

Titrations of Chloroperoxidase with Phosphines. Although dimethyl- and diethylphenylphosphine have been used in our previous studies of P-450[18] and in studies by Ullrich and coworkers,[12,14,17] the affinities of these phosphines for chloroperoxidase and their solubilities in water were too low to form homogeneous adducts. Thus, we have used a more soluble (hydrophilic) and less bulky phosphine, bis(hydroxymethyl)methylphosphine, as a ligand for both ferric and ferrous chloroperoxidase as well as for P-450-CAM. As shown in Figure 1, ferric and ferrous chloroperoxidase undergo spectral changes upon addition of the phosphine with single sets of isosbestic points. This indicates that a single phosphine adduct is formed in each case without any detectable intermediates. Double-reciprocal plots of the titration results in both cases yield straight lines with x intercepts of –0.5 and –0.13 mM^{-1}, respectively (Figure 1, insets). Hill plots (not shown) of these results give a straight line with the slope of unity (n = 1) in both cases. Thus, both ferric and ferrous chloroperoxidase form complexes with the phosphine in a 1:1 molar ratio with dissociation constants (K_d) of 2.0 and 7.7 mM, respectively, under the conditions employed. Establishment of a 1:1 binding stoichiometry is especially important for the ferrous adduct since it has recently been reported that a bis(phosphine)ferrous porphyrin model complex also exhibits an unusually red-shifted Soret maximum.[25]

In the case of the ferric enzyme (Figure 1A), phosphine binding converts the single broad Soret peak of the native enzyme at 399 nm to two peaks (376 and 450 nm) with nearly equal intensity. Concomitantly, the high-spin-type spectral features in the visible region (peaks at 514, 544, 590, and 650 nm) change to those of a low-spin-type complex with a single band around 553 nm. This spectral change is very similar to those observed upon thiol binding to native ferric chloroperoxidase[11] and ferric P-450[15,16] or upon phosphine binding to ferric P-450[12,17] and trans to thiolate in ferric heme iron model complexes.[14] By way of contrast, phosphine

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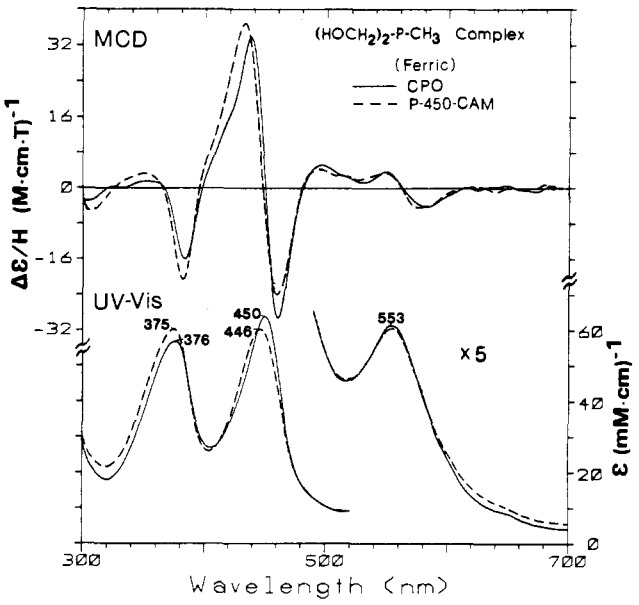

Figure 2. MCD (top) and UV–visible absorption (bottom) spectra of the ferric chloroperoxidase (solid line) and P-450-CAM (dashed line) complexes with bis(hydroxymethyl)methylphosphine. The spectra were obtained at 4 °C with 10–15 μM enzyme concentrations in 0.1 M potassium phosphate buffer, at pH 6.5 with 32 mM phosphine for chloroperoxidase and at pH 7.3 with 16 mM phosphine in the presence of 6.6 μM *d*-camphor for P-450-CAM. See text for further details.

binding trans to histidine in ferric myoglobin and horseradish peroxidase produces complexes with single Soret maxima at 427 nm.[26]

With ferrous chloroperoxidase (Figure 1B), a new, red-shifted, relatively intense Soret peak appears at 457.5 nm upon phosphine binding. In the visible region, the broad peak at 552 nm changes to a more complex pattern having distinct peaks at 551 and 578 nm with shoulders evident on both sides of the 551-nm peak. From the uniform spectral changes caused by the stepwise additions of the phosphine to the ferrous enzyme and from the above-mentioned establishment of a 1:1 binding stoichiometry, the presence of multiple species can be ruled out as the origin of the increased complexity of the UV–visible absorption spectrum of the phosphine adduct.

Comparison of UV–Visible Absorption and MCD Spectra of the Phosphine Complexes of Ferric and Ferrous Chloroperoxidase and P-450. In order to further characterize the unique spectral properties of the phosphine complexes of ferric and ferrous chloroperoxidase, we have generated analogous phosphine adducts of P-450-CAM. In Figure 2, the UV–visible absorption and MCD spectra of the phosphine-bound derivatives of ferric chloroperoxidase and P-450-CAM are overplotted. Neither phosphine adduct has been previously studied with MCD spectroscopy. The pattern of MCD bands for these species clearly falls into a category that includes ferrous–CO P-450 and its models[27] and thiolate-bound ferric P-450-CAM[16] and chloroperoxidase,[11] all of which exhibit characteristic hyperporphyrin spectra. With ferric model heme complexes having at least one biomimetic axial ligand, hyperporphyrin spectra have only been observed when one of the axial ligands is a thiolate.[14] Taken together, these facts provide strong support for the presence of an endogenous thiolate ligand coordinated to the heme iron of ferric chloroperoxidase.

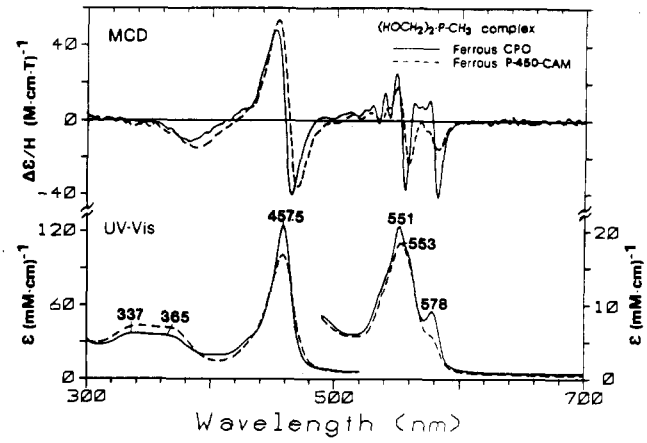

Figure 3. MCD (top) and UV–visible absorption (bottom) spectra of the ferrous chloroperoxidase (solid line) and P-450-CAM (dashed line) phosphine complexes. Experimental conditions were the same as those given in the legend to Figure 2, except that nearly stoichiometric amounts of dithionite were added to the ferric sample to reduce the enzymes and that the spectra of the chloroperoxidase adduct was obtained in the presence of 100 mM phosphine at pH 7.5. Under these conditions (4 °C) the adducts were stable for at least 1 h, and no significant conversion to P-420 forms was detected for either enzyme. In order to be certain that the UV–visible absorption spectra of the enzyme solutions were free of interference from excess amounts of dithionite (λ_{max} = 315 nm), small volumes of air were gently bubbled through the sample solutions until no further decreases in A_{315} were observed, during which time the Soret peak position (457.5 nm) and intensity remained unchanged. Thus the two small peaks in the 300–360-nm region for both enzymes are intrinsic to the ferrous enzyme–phosphine complexes.

A similar comparison between the phosphine adducts of ferrous chloroperoxidase and P-450-CAM with UV–visible absorption and MCD spectroscopy is shown in Figure 3. The adduct of ferrous P-450-CAM with bis(hydroxymethyl)methylphosphine exhibits a UV–visible absorption spectrum that is nearly identical with that observed previously for the ferrous enzyme adduct with dimethylphenylphosphine[18] except that the α peak around 580 nm for the former is less distinct than that for the latter. Extensive investigations of ligand adducts of ferrous P-450 have led to the inclusion of the phosphine complex in the hyperporphyrin category.[18] As the UV–visible absorption spectrum of the phosphine adduct of ferrous chloroperoxidase has a much more distinct α peak (578 nm) than does phosphine-bound ferrous P-450-CAM, small differences are also observed in this region of the MCD spectra of these species (Figure 3, top). Overall, the MCD spectra of the two phosphine adducts are quite similar in both the Soret and the visible regions, except that the spectrum of the P-450-CAM derivative in the visible region has somewhat less developed features than those of the chloroperoxidase derivative. Althogether, the close similarity between the MCD spectra of the phosphine complexes of these two enzymes strongly suggests the presence of identical ligand coordination structures in both enzyme adducts, i.e. RS–ferrous heme–phosphine.

EPR Properties of the Phosphine Adducts of Ferric Chloroperoxidase and P-450. In order to see whether the close spectral similarities described above are also seen with other spectroscopic techniques, the EPR parameters (*g* values) of the phosphine adducts of the two enzymes have been compared (Table I). No high-spin signals are detected at 77 K from the phosphine adducts of either enzyme, consistent with the low-spin-type UV–visible absorption spectral character of these derivatives at ambient temperature (Figure 2). In their native ferric states, likely as a result of differences in the endogenous sixth ligands to the low-spin heme iron, the enzymes exhibit distinguishable sets of *g* values:[6,28] the distance (Δg) between the highest (g_1) and the lowest (g_3) *g* values for chloroperoxidase (Δg = 0.80) is considerably larger

(26) Dimethylphenylphosphine also binds to ferric myoglobin and horseradish peroxidase to yield complexes with phosphine phosphorus and histidine nitrogen as the axial donor atoms. However, the resulting complexes each exhibit a UV–visible absorption spectrum [λ, nm (ε, mM^{-1} cm^{-1}): 373 (~35), 427 (~75), 537 (~10) in 0.1 M potassium phosphate at pH 7.0 and 4 °C] having a single Soret peak that is much less red-shifted than is observed for the phosphine adducts of chloroperoxidase and P-450.

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Table I. EPR Parameters (g Values) of Low-Spin Phosphine Complexes of Ferric Chloroperoxidase and P-450[e]

heme protein derivatives	g_1	g_2	g_3	ref
Chloroperoxidase				
native (pH 6)	2.63	2.26	1.83	Hollenberg et al.[6] this work
+bis(hydroxymethyl)-methylphosphine[a]	2.59	2.29	1.82	this work
P-450-CAM				
native (CAM-free)	2.44	2.25	1.91	Sono et al.[16]
+bis(hydroxymethyl)-methylphosphine[b]	2.51	2.28	1.86	this work
+dimethylphenylphosphine[c]	2.51	2.28	1.86	this work
+diethylphenylphosphine	2.50	2.28	1.88	Ruf et al.[14]
P-450-LM[d]				
+diethylphenylphosphine	2.49	2.28	1.88	Mansuy et al.[17]

[a] With ~80 μM enzyme and 50 mM phosphine at pH 6. [b] With ~50 μM enzyme, 50 mM phosphine, and 30 μM camphor at pH 7. [c] With ~80 μM enzyme, 1 mM phosphine, and 180 μM camphor at pH 7. [d] Rat liver microsomal enzyme was used. [e] All values were determined at liquid-nitrogen temperature (77 K). The measurements in this work were carried out in 0.1 M potassium phosphate buffer under the following instrumental conditions: modulation amplitude, 10 G; microwave power, 20 mW; microwave frequency, ~9.02 GHz; modulation frequency, 100 kHz.

than that for substrate-free native ferric P-450-CAM ($\Delta g = 0.53$). Upon ligation of the phosphine to the heme iron of these enzymes, however, the spread in g values becomes much smaller: Δg values are 0.77 and 0.65 for chloroperoxidase and P-450-CAM, respectively. In addition, the actual g values of the phosphine adducts of the two enzymes (Table I) are more nearly identical with each other and feature noticeable shifts in the middle g value (g_2) to lower field relative to those of native chloroperoxidase (2.26 → 2.29) and P-450-CAM (2.25 → 2.28).[17] Thus, the EPR properties of the phosphine adducts of ferric chloroperoxidase and P-450, in combination with the other spectroscopic data presented in this paper, provide positive support for the presence of essentially identical coordination structures in the two enzymes.

CD Spectral Properties of the Phosphine Adducts of Chloroperoxidase and P-450-CAM. In Figure 4, we report the CD spectra of the ferric and ferrous adducts of chloroperoxidase and P-450-CAM with bis(hydroxymethyl)methylphosphine. The CD spectra of the phosphine complexes of ferric and ferrous P-450-CAM reported in this work are very similar to those of the respective dimethylphenylphosphine complexes.[18,29] Thus, different phosphines have relatively small effects on the observed spectra. The only CD spectra of chloroperoxidase derivatives to have been reported previously are those for the native ferrous (high spin) enzyme and its CO complex.[30] The CD spectrum of ferrous–CO chloroperoxidase between 450 and 700 nm is similar to that of ferrous–CO P-450-CAM reported by Peterson,[31] while the native ferrous forms of these enzymes exhibit different CD spectra. For the phosphine complexes studied in the present work, the CD spectra of the ferric chloroperoxidase and P-450-CAM derivatives are clearly distinguishable (Figure 4A). This is in contrast to the close similarities in UV–visible absorption, MCD, and EPR properties of phosphine-bound ferric chloroperoxidase and P-450-CAM reported herein. Nonetheless, the CD spectra do have a common feature in the 400–500 nm region where both consist of two transitions: a peak (437 nm) and a trough (460 nm) in the chloroperoxidase case and two troughs (419 and 454 nm) for P-450-CAM. In previous papers reporting the CD spectra of low-spin ferric[29] and ferrous[18] complexes of P-450-CAM with various exogenous ligands, we have observed that hyperporphyrin

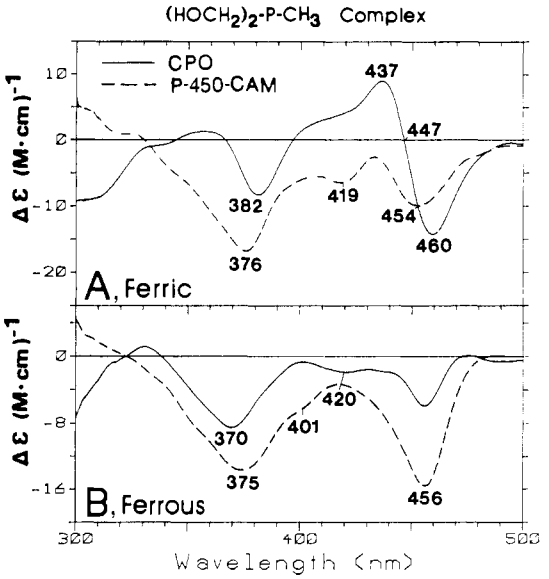

Figure 4. CD spectra of the phosphine adducts of ferric (A) and ferrous (B) chloroperoxidase (solid line) and P-450-CAM (dashed line) in the Soret region. See the captions to Figures 2 and 3 for experimental conditions.

complexes display CD spectra with two transitions in the Soret region such as is seen here (Figure 4A). Hyperporphyrin spectra have been suggested to originate from an orbital mixing mechanism wherein the doubly degenerate Soret π–π^* transitions are split into two components by mixing with a cysteine sulfur to porphyrin π^* charge-transfer transition.[10] Myer's idealized theory for the origin of CD spectra of heme proteins[32,33] also rpedicts that loss of the inherent degeneracy of the Soret transition should result in a CD spectrum with two Soret region transitions that develop signal intensity and sign independently. Thus, the hyperporphyrin case fulfills the condition for loss of the inherent degeneracy of the Soret transition and is expected to exhibit a CD spectrum with two transitions in the Soret (400–500-nm) region. This interpretation can be applied to the present case of chloroperoxidase. In addition, it is interesting to note that the CD spectrum seen here for the phosphine complex of *ferric* chloroperoxidase (Figure 4A) is quite similar to that of *ferrous*–CO P-450-CAM,[18,31] a typical hyperporphyrin adduct.

In the ferrous case, the CD spectra of both enzyme–phosphine adducts resemble each other and consist of two major troughs around 370 and 455 nm. An additional weak and less well-resolved trough is observed for both adducts at 420 nm for chloroperoxidase and around 400 nm for P-450-CAM. Thus, both CD spectra have two transitions in the 400–500-nm region as is to be expected for hyperporphyrins.[18] Curiously, the CD spectrum of the phosphine adduct of *ferrous* chloroperoxidase (Figure 4B, solid line) resembles that of the benzylmercaptan complex of *ferric* P-450-CAM, a bis(thiolate)-type hyperporphyrin complex previously studied by our laboratory.[16,29]

Conclusions

Characteristic UV–visible absorption and MCD spectral features that appear upon phosphine ligation to ferric and ferrous chloroperoxidase bear marked similarities to those observed with analogous derivatives of cytochrome P-450. The resultant spectra clearly fall in the hyperporphyrin classification that can also be generated upon ligation of CO to the ferrous form or of a thiolate ligand to the ferric form of these two enzymes. In this respect, phosphines are multifunctional ligands as compared with CO and thiolate in that they may serve as ligands to both the ferric and ferrous heme iron of these enzymes. Since the generation of a hyperporphyrin spectrum **requires** the presence of a thiolate axial

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ligand trans to CO in the ferrous form[10] or trans to thiolate or phosphine in the ferric form,[14] the results observed in this study of phosphine binding, together with the previous findings with CO and thiolate, strongly support the conclusion that the heme iron of chloroperoxidase has an endogenous thiolate axial ligand. Given the well-established presence of an endogenous thiolate ligand to the heme iron of P-450, this conclusion is further strengthened by the close similarities observed between the UV-visible absorption, MCD, and, for ferric forms, EPR spectra of the phosphine adducts of chloroperoxidase and P-450. The spectroscopic evidence for thiolate ligation to chloroperoxidase stands in contrast to the chemical evidence against such a ligand.[2]

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Registry No. Cytochrome P-450, 9035-51-2; bis(hydroxymethyl)-methylphosphine, 5958-52-1; dimethylphenylphosphine, 672-66-2; chloroperoxidase, 9055-20-3; heme, 14875-96-8; iron, 7439-89-6; cysteine, 52-90-4.

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New Multidentate Ligands. 27. Synthesis and Evaluation of Metal Ion Affinities of New Endocyclic Hydroxamate Macrocycles

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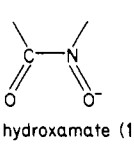
Two synthetic pathways for the synthesis of endocyclic polyhydroxamate macrocycles are explored: peroxomolybdate oxidation of endocyclic amides and the cyclization of benzyl-protected hydroxamic acid intermediates. Because of the apparent sensitivity of the peroxomolybdate oxidation step to steric effects, the benzylhydroxamate route was found to be superior, with the smallest stepwise yield being the ring closure (~26%) and with good yields of other reaction steps up to the 80-90% range, for an overall yield of 2% based on the triethylene glycol starting material. The two endocyclic dihydroxamate macrocycles synthesized, the sexidentate 5,14-dihydroxy-4,15-dioxo-1,5,14,18-tetraaza-8,11,21,24-tetraoxacyclohexacosane and its octadentate 1,18-diacetic acid derivative, were characterized by potentiometric measurement of their proton, Ni(II), and Fe(III) affinities. The results show stability enhancement over analogous complexes having exocyclic hydroxamate donor groups.

Introduction

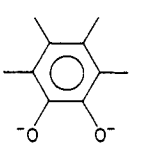
There is considerable interest in the development of new selective ligands for the formation of iron(III), gallium(III), and indium(III) complexes of high stability, particularly for the treatment of iron overload disease (Cooley's anemia)[1-4] and for the imaging of tumors and organs in the human body.[5] Considerable attention has also been given to the siderophores,[6] which are microbial hydroxamate and catecholate ligands having high affinity for iron(III) and other trivalent metal ions. Synthetic ligands containing three bidentate catecholate donor groups have been synthesized as models of the siderophores and have been found to have high affinities for iron(III).[4] Thus far no synthetic hydroxamate ligands having iron(III) affinities comparable to those of the natural trihydroxamate siderophores have been reported.

The structures of the natural siderophores vary considerably with respect to the placement of the bidentate hydroxamate or catecholate donor groups. The hydroxamate siderophores contain functional groups in acyclic, exocyclic, and endocyclic arrangements.[6] Of these, the endocyclic ligands, such as deferriferrioxamine E, show the highest affinities for iron(III), because of the more effective operation of the macrocyclic effect in this type of structure. Thus far no natural endocyclic catecholate siderophores have been identified. Enterobactin, which has the highest iron(III) affinity of any ligand yet measured,[4] has three exocyclic catecholamide donor groups. Reports are now appearing, however, on the design and synthesis of endocyclic catecholate ligands modeled after the siderophores.[2,7-9] On the other hand, synthetic endocyclic trihydroxamates have thus far not been reported.

In addition to the macrocyclic effect, the synthesis and study of endocyclic hydroxamates and catecholates are of interest because of the specificities that can be built into the structure by variation of ring size. The structures of the hydroxamate and catecholate donor groups, indicated by **1** and **2**, place stringent



hydroxamate (1)



catecholate (2)

demands on the substituent polyatomic groups that link them together in such a manner as to place six negative oxygens symmetrically around an octahedral metal ion. The trigonal nature of these functional groups demands that the bridging groups approach from the back side for hydroxamate and from the back side or at the 3,6-positions for the catecholates. The use of molecular models and comparison with natural siderophores indicate that connecting chains of eight atoms or more are needed

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