Reaction of Molecular Oxygen with Pyridinehemochromes in Aqueous Solution[1]

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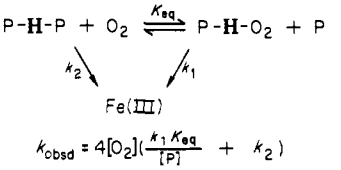
Abstract: The variation of the electronic spectrum of aqueous protoheme with the pyridine concentration in the presence and absence of cetyltrimethylammonium bromide micelles was used to establish the nature of pyridine hemochromes over the entire ligand concentration range. Excess pyridine was found to initially convert aqueous protoheme to a dipyridine heme dimer which does not fully monomerize until the pyridine concentration exceeds 2–3 M. The rate of reaction of this dimeric species with molecular oxygen was examined in 0.05 M boric acid buffer as a function of the pyridine concentration. The observed pseudo-first-order rate constant (0.3 s^{-1} at $[O_2]$ = 213 μM) was independent of the pyridine concentration at pH 8.5 from 0.001–0.2 M. At higher pH values, the rate of reaction increased and a dependence on the reciprocal of the pyridine concentration appeared. The magnitude of this inverse dependence increased with increasing pH. These data are interpreted solely in terms of an outer-sphere oxidation mechanism and are compared to results obtained previously for the oxidation of iron(II) porphyrins in amine solvents and in aqueous tris buffer.

Introduction

The interaction of molecular oxygen with ferrohemochromes has been a subject of intense interest because of the modulating influence that hemoproteins exert on oxyheme complexes.[2] Though several elegant models for the active site of myoglobin[3-9] and hemoglobin[10] have recently been prepared in aprotic media, the redox chemistry of iron(II) porphyrins in aqueous solution is still unknown. The only investigation of ferrohemochrome oxidation in aqueous solution is the early work of Wang,[11] who suggested the mechanism described in Scheme I for the reaction of 5×10^{-5} M dipyridineprotoheme with molecular oxygen in 0.1 tris buffer at pH 8.5, where P stands for pyridine and P–H–P and P–H–O_2 represent dipyridineprotoheme and monopyridineoxyprotoheme, respectively.[12] The major path of the reaction was thought to proceed via an inner-sphere mechanism (k_1) involving an oxyheme intermediate, with the outer-sphere process (k_2) becoming important only at high pyridine concentrations. Though

Scheme I



$$k_{obsd} = 4[O_2]\left(\frac{k_1 K_{eq}}{[P]} + k_2\right) \quad (1)$$

no oxygenated heme complex was detected, the observation of an inverse order in pyridine in the concentration range 0.1–0.5 M was cited as support for this dual pathway mechanism.[11]

The demonstration by Smith[13] that pyridine concentrations of 2–10 M are required to fully monomerize 10^{-5} M aqueous protoheme raises questions about the nature of the reacting species in the above experiments. Thus, it is highly unlikely that 5×10^{-5} M protoheme is monomeric in 0.1 M aqueous pyridine if 1×10^{-5} M protoheme is not monomeric until 2–10 M pyridine.[14]

We have confirmed that excess pyridine does not convert aqueous protoheme to the hexacoordinate monomer except at pyridine concentrations above 2 M. At lower concentrations, the diligated heme dimer is formed instead with a binding constant of 3×10^5 M^{-2}. We report further that the oxidation of this dimer by molecular oxygen in 0.05 M boric acid buffer at pH 8.5 is *independent* of the pyridine concentration and proceeds entirely via an outer-sphere process without the intermediacy of an oxyheme complex.

Experimental Section

Materials. Pyridine (Aldrich, spectral grade) was distilled and stored over Linde 4A molecular sieves prior to use. Sodium dithionite (Aldrich) was used without further purification. Small vials containing about 1 g of sodium dithionite were prepared, sealed with Parafilm, and stored in a desiccator. Once opened, the unused contents of a vial were discarded. If a vial contained lumps or smelled of sulfur dioxide, its contents were discarded. These precautions were taken because sodium dithionite is hygroscopic and may decompose upon prolonged exposure to moist air.

Crystalline, bovine hemin chloride (Aldrich or Eastman) was characterized by the pyridine hemochrome test, according to the method of Paul et al.,[15] prior to use. Only hemin stock solutions which yielded α-maximum/α-minimum ratios of 3.40–3.50 were utilized (literature value 3.46).[16] Nitrogen gas (ppm grade) was used to deoxygenate the

(1) Abstracted from a Ph.D. Thesis submitted by R.A. to the Graduate School of the Polytechnic Institute of New York, 1980.

(2) (a) C. E. Castro, *J. Theor. Biol.*, **33**, 475 (1971); (b) C. E. Castro, *Porphyrins*, **5**, 1 (1978).

(3) (a) C. K. Chang and T. G. Traylor, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2647 (1973); (b) C. K. Chang and T. G. Traylor, *J. Am. Chem. Soc.*, **95**, 5810 (1973); (c) C. K. Chang and T. G. Traylor, *ibid.*, **95**, 8477 (1973); (d) W. S. Bringar and C. K. Chang, *ibid.*, **96**, 5595 (1974).

(4) J. E. Baldwin and J. Huff, *J. Am. Chem. Soc.*, **95**, 5757 (1973).

(5) (a) G. C. Wagner and R. J. Kessner, *J. Am. Chem. Soc.*, **96**, 5593 (1974); (b) D. L. Anderson, C. J. Wechsler, and F. Basolo, *ibid.*, **96**, 5599 (1974); (c) J. E. Baldwin et al., *ibid.*, **96**, 5600 (1974).

(6) (a) J. E. Baldwin et al., *J. Am. Chem. Soc.*, **97**, 226 (1975); (b) J. Almog, J. E. Baldwin, and J. Huff, *ibid.*, **97**, 227 (1975); (c) J. P. Collman et al., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1326 (1974); (d) J. P. Collman et al., *J. Am. Chem. Soc.*, **96**, 6522 (1974); (e) J. P. Collman et al., *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3333 (1976); (f) J. P. Collman et al., *J. Am. Chem. Soc.*, **97**, 7185 (1975).

(7) (a) J. P. Collman et al., *J. Am. Chem. Soc.*, **95**, 7868 (1973); (b) J. P. Collman, R. R. Gagne, and C. A. Reed, *ibid.*, **96**, 2629 (1974); (c) J. P. Collman et al., *ibid.*, **97**, 1427 (1975).

(8) For reviews citing much of the work in ref 3–7, see: (a) J. P. Collman, *Acc. Chem. Res.*, **7**, 265 (1977); (b) B. R. James, *Porphyrins*, **5**, 207 (1978); (c) C. K. Chang and D. Dolphin, *Bioorg. Chem.* **4**, 37 (1978); (d) T. G. Traylor, ibid., **4**, 437 (1978).

(9) (a) A. R. Battersby et al., *J. Chem. Soc., Chem. Commun.*, 879 (1976); (b) J. E. Baldwin, T. Klose, and M. Peters, *ibid.*, 881 (1976); (c) J. E. Kagan, D. Mauzerall, and R. B. Merrifield, *J. Am. Chem. Soc.*, **99**, 5485 (1977); (d) C. K. Chang, *ibid.*, **99**, 2819 (1977); (e) D. A. Buckingham, M. J. Gunter, and L. N. Mander, *ibid.*, **100**, 2899 (1978).

(10) (a) J. Geibel, C. K. Chang, and T. G. Traylor, *J. Am. Chem. Soc.*, **97**, 5924 (1975); (b) J. Cannon, J. Geibel, M. Whipple, and T. G. Traylor, *ibid.*, **98**, 3395 (1976); (c) J. Geibel, J. Cannon, D. Campbell, and T. G. Traylor, *ibid.*, **100**, 3575 (1978); (d) T. G. Traylor et al., *J. Chem. Soc., Chem. Commun.*, 732 (1977).

(11) O. H. W. Kao and J. H. Wang, *Biochemistry*, **4**, 343 (1965).

(12) The factor 4 in eq 1 accounts for the fact that four hemes are oxidized by each molecule of oxygen as it is reduced to water.

(13) M. H. Smith, *Biochem. J.*, **73**, 90 (1959).

(14) See also the conflicting report by W. A. Gallagher and W. B. Elliott, *Biochem. J.*, **97**, 187 (1965), where it is suggested that at least in media of low ionic strength, pyridine hemochrome exists as a monomeric species even with pyridine concentrations as low as 5×10^{-3} M.

(15) K.-Paul, H. Theorell, and A. Akeson, *Acta Chem. Scand.*, **7**, 1284 (1953).

(16) J. H. Fuhrhop and K. M. Smith, *Porphyrins Metalloporphyrins*, 804 (1975).

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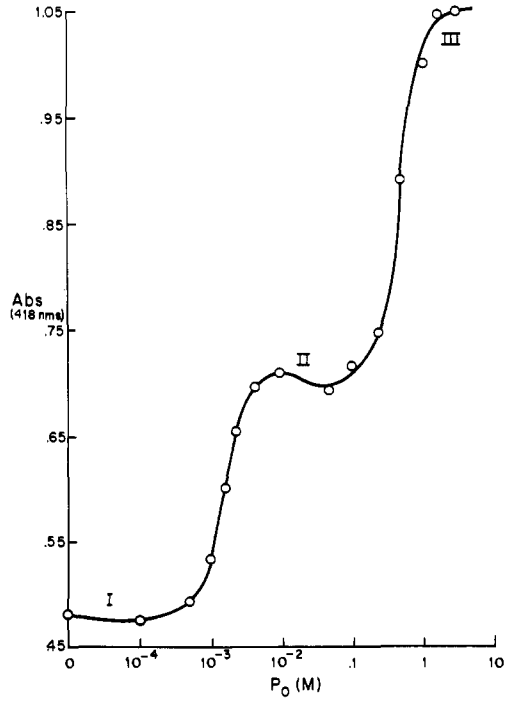

Figure 1. Spectrophotometric titration of 10^{-5} M protoheme by pyridine in 0.05 M boric acid buffer at pH 8.5 and 25 °C. Absorbance was monitored at 418 nm.

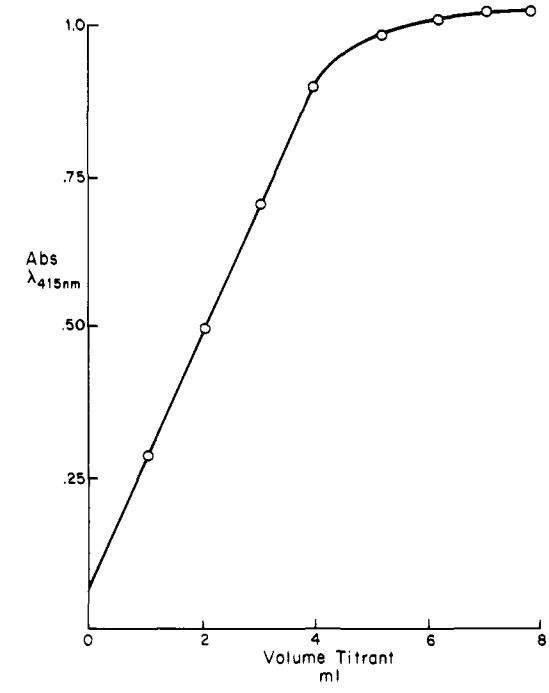

Figure 2. Titration of 10^{-5} M hemin chloride with a 0.04% (w/w) sodium dithionite solution in 0.05 M boric acid buffer at pH 8.5 and 0.1 M pyridine. Absorbance was monitored at 415 nm.

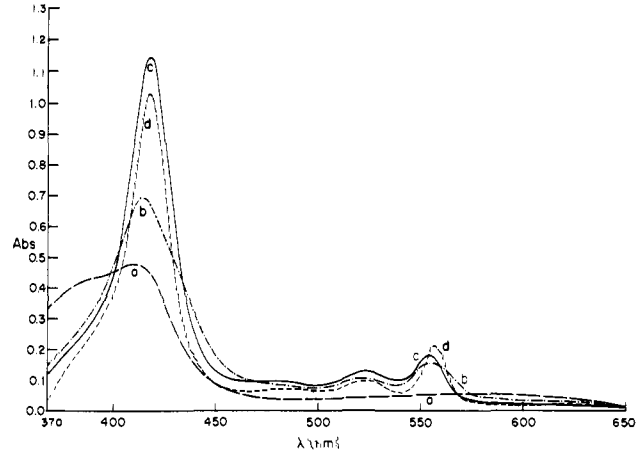

Figure 3. Electronic spectra of 10^{-5} M protoheme in 0.05 M boric acid buffer at pH 8.5 and 25 °C with pyridine concentration of (a) 0–0.001 M (—,—), (b) 0.006–0.1 M (·-·-), (c) 1 M (—), (d) 0.01 M with [CTAB] = 0.05 M (- - -).

hemochrome solutions prior to reaction.

Instrumental. All electronic spectra were taken on a Cary 14 spectrophotometer. Oxygen concentrations were determined with a Yellow Springs Clark type membrane-covered oxygen-sensing electrode, using a Yellow Springs YSI 5352 membrane. The electrode was calibrated with oxygen-saturated, distilled, deionized water at 0.0 °C.

The reaction of pyridine protohemes with molecular oxygen was monitored with an Aminco-Morrow or a Durrum model D-100 stopped-flow spectrophotometer. The temperature was held constant to ±0.1 °C by a Bayley Instruments Model 238 precision temperature controller used in conjunction with a circulating water bath.

Spectrophotometric Titration. Fifteen solutions, 10^{-5} M in hemin chloride and 0.05 M in boric acid buffer at pH 8.5, were prepared, containing varying concentrations of pyridine between 0 and 3.0 M. Each solution was reduced with 1.0 mg of sodium dithionite/mL of solution, and its electronic spectrum was immediately recorded between 350 and 650 nm. The titration curve in Figure 1 was obtained by plotting the absorbance at 418 nm in the Soret band as a function of the pyridine concentration. The stoichiometry and binding constant of complex II were determined by the Hildebrand–Benesi method,[17] using absorbance data at 412.5 nm.

Kinetic Studies. A 100-mL three-necked flask equipped with a serum cap, a 100 mL dropping funnel with an equalizing arm, and a gas outlet tube were charged with 25.0 mL of a 2×10^{-5} to 1×10^{-4} M ferrihemochrome solution containing varying quantities of pyridine in 0.05 M boric acid buffer adjusted to the desired pH. The dropping funnel was then filled with a 0.04% (w/w) buffered sodium dithionite solution of the same pH and pyridine concentration. The top of the dropping funnel was fitted with a gas inlet tube through which nitrogen was admitted to the system. After deoxygenation of the hemin solution for 20 min, a set quantity of the dithionite (2–8 mL) was added to the system, and the solution was stirred for 5 min. The exact amount of dithionite required to reduce the ferrihemochrome completely was determined by prior titration. The absorbance of the hemin solution at 415 nm was monitored as a function of the volume of titrant (Figure 2). In general, the higher the pyridine concentration, the lower the quantity of dithionite needed for complete reduction. Conditions were then chosen so that 75% of the hemin was reduced to ensure that no excess active reducing agent would remain in the reaction mixture. The final heme concentration varied from 6×10^{-6} to 3×10^{-5} M.

The reduced solution was drawn into one of the syringes of the stopped-flow spectrophotometer via a long needle, equipped with a Luer-Lock fitting, which was inserted through the serum cap into the hemochrome solution. The other syringe was filled with a buffered solution of known oxygen concentration containing an identical ligand concentration. After temperature equilibration, the two solutions were rapidly mixed, and the reaction was monitored at 420 or 560 nm. The resultant curves were analyzed by assuming pseudo-first-order conditions in hemochrome,[11] and observed rate constants were determined by least-squares linear regression on a Texas Instruments 980A minicomputer. Correlation coefficients were generally about 0.99, and the error is estimated to be about 20%. The rate constants obtained did not depend on the final heme concentration,[18] on the monitoring wavelength, or on the quantity of reducing agent used below 100% reduction.

Results

The Nature of the Pyridine Hemochrome Complexes. A spectrophotometric titration of 10^{-5} M protoheme with pyridine was performed in boric acid buffer at pH 8.5 in an attempt to confirm the nature of the hemochrome complexes in solution. The

(17) H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.*, **71**, 2703 (1949).

(18) In the pyridine concentration range 0.001–0.2 M above 0.2 M pyridine that heme concentration did affect the kinetics as is explained in the Results and Discussion.

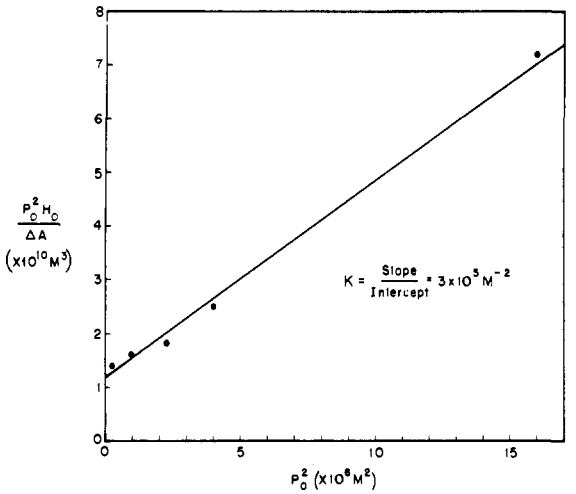

Figure 4. Hildebrand–Benesi plot describing the binding of two pyridine molecules to I to form II according to eq 2.

existence of three distinct heme complexes (I–III) can be inferred from the titration curve of Figure 1.

Below 0.001 M pyridine, the electronic spectrum of the protoheme solution (Figure 3a) resembles that obtained in the absence of any external ligand. Complex I is, therefore, presumed to be the diaquoheme dimer.[19] Between 0.001 and 0.006 M pyridine, complex II is formed and it persists up to 0.1 M pyridine. The rather broad, diffuse α and β bands in the electronic spectrum of II (Figure 3b) exhibit λ_{max} values of 553 and 522 nm, respectively, and indicate the presence of coordinated pyridine. The Soret band is only of moderate intensity with λ_{max} at 413 nm. The peak intensities and λ_{max} values exhibited by this complex do not agree with those given in the literature for dipyridineferroprotoheme.[16]

As the pyridine concentration is raised above 0.1 M, complex III begins to form and becomes the exclusive species in solution above 2 M pyridine. The electronic spectrum of III exhibits a substantial increase in intensity in the Soret band with a shift of λ_{max} to 418 nm (Figure 3c). The α and β bands sharpen considerably, but shift only slightly to 554 and 523 nm, respectively. This spectrum agrees with the literature data reported for the monomeric hexacoordinate dipyridine hemochrome.[16]

The dimeric nature of II is indicated by the fact that it is not observed as an intermediate when protoheme is titrated with pyridine in methylene chloride, where the starting heme is monomeric. Furthermore, aqueous cetyltrimethylammonium bromide (CTAB) micelles, which are known to monomerize heme complexes,[20] convert the Soret band of II (Figure 3b) to that of the hexacoordinate pyridine hemochrome, III (see Figure 3d).

The stoichiometry of complex II was determined by using the Hildebrand–Benesi method.[17] The treatment which assumes a 1:1 stoichiometry of pyridine to I was totally unsatisfactory. However, if a dipyridine complex is assumed to be the only ligated species in solution, eq 2 is obtained, where P_0 and H_0 are the total

$$\frac{P_0^2 H_0}{\Delta A} = \frac{P_0^2}{\Delta \epsilon} + \frac{1}{K \Delta \epsilon} \qquad (2)$$

pyridine and heme concentrations, ΔA and $\Delta \epsilon$ are the changes in absorbance and extinction coefficient (at 412.5 nm) caused by complexation, and K is the binding constant. Figure 4 shows that a plot of $P_0^2 H_0/\Delta A$ vs. P_0^2 yields a good linear relationship. The intermediate complex, II, can therefore be identified as a dipyridine heme dimer. The equilibrium constant for formation of II was determined from the slope and intercept of the plot in Figure 4 as 3×10^5 M^{-2}.

Scheme II

$$\underset{\text{I}}{H_2O\text{-}H_2\text{-}OH_2} \underset{\text{0–0.001 M}}{} \rightleftharpoons^{+2P}_{-2P} \underset{\text{II}}{P\text{-}H_2\text{-}P} \underset{\text{0.006–0.1 M}}{} \rightleftharpoons^{+2P}_{-2P} \underset{\text{III}}{2P\text{-}H\text{-}P} \underset{\text{>2 M}}{}$$

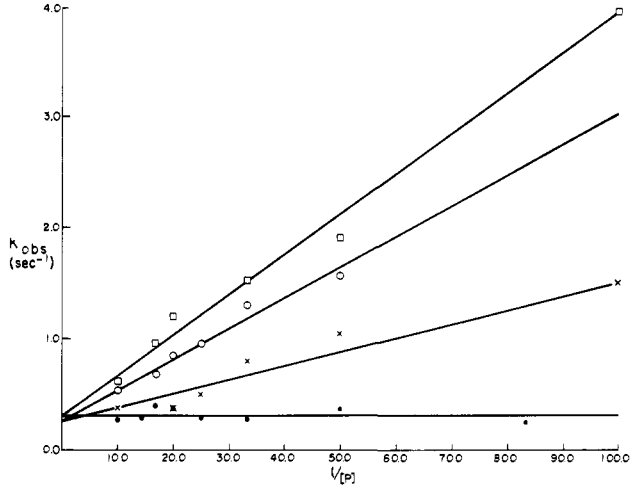

Figure 5. Plots of the observed pseudo-first-order rate constant for hemochrome oxidation as a function of the reciprocal of the pyridine concentration at four different pH values: (●) pH 8.5; (×) pH 9.0; (○) pH 9.5; (□) pH 10.0. All conditions were 0.05 M boric acid buffer, $[O_2]$ = 213 μM, and temp = 25.0 °C.

A summary of the equilibria operating in solution and the pyridine concentration ranges in which each complex is stable is provided in Scheme II.

The Kinetics of Hemochrome Oxidation. The oxidation of protoheme by molecular oxygen was performed with thirty different pyridine concentrations between 0 and 3 M in 0.05 M boric acid buffer at pH 8.5 and 25.0 °C. Below 0.001 M pyridine, the reaction proceeded too rapidly and no stopped-flow trace could be detected. As the pyridine concentration was raised above 0.001 M, a curve became visible which increased in amplitude with the pyridine concentration up to about 0.006 M. Increasing the pyridine concentration above 0.006 M had no further effect on the amplitude of the stopped-flow trace. From the point at which the oxidation process was first detected (0.0014 M pyridine) up to about 0.2 M, the observed rate constant (0.3 ± 0.06 s^{-1} at $[O_2]$ = 213 μM) was *independent* of the pyridine concentration.

Between 0.2 and 2 M pyridine, the kinetics became more complex with two distinct processes becoming evident. The precise point at which this transformation in kinetic behavior occurred depended on the heme concentration. The higher the heme concentration, the more pyridine was required to induce this change in kinetics. For example, a single first-order process (k_{obsd} = 0.3 s^{-1}) could be detected only up to 0.2 M pyridine at a heme concentration of 8×10^{-6} M but up to 0.4 M pyridine at a heme concentration of 3×10^{-5} M. At pyridine concentrations above 2 M, a single first-order oxidation process was again observed but with a reduced rate constant of 0.13 s^{-1} ($[O_2]$ = 213 μM).

The kinetics of oxidation were also examined as a function of pH in the pyridine concentration range of 0.02–0.1 M. The results are shown in Figure 5, as plots of k_{obsd} vs. the reciprocal of the pyridine concentration. As the pH was raised, the overall oxidation rate *increased* and an inverse order in pyridine appeared. The slopes of the plots in Figure 5 increased with increasing pH, but the intercepts remained constant throughout. Interestingly, the slopes of the lines are not directly related to the hydroxide ion concentration but to the concentration of $[H_2BO_3^-]$ as demonstrated by Figure 6.

Discussion

The data obtained from the spectrophotometric titration confirm the observation of Smith[13] that excess pyridine does not convert

(19) (a) J. Shack and W. M. Clark, *J. Biol. Chem.*, **171**, 143 (1947); (b) J. N. Phillips, *Rev. Pure Appl. Chem.*, **10**, 35 (1960); (c) W. I. White, *Porphyrins*, **5**, 303 (1978).

(20) (a) J. N. Phillips, *Curr. Trends Heterocycl. Chem. Proc. Symp.*, 30 (1958); (b) B. Dempsey, M. B. Low, and J. N. Phillips, *Proc. Int. Congr. Biochem.*, **5**, 1959 (1961); (c) J. N. Phillips, *Compr. Biochem.*, **9**, 34 (1963); (d) R. J. Porra and O. T. G. Jones, *Biochem. J.*, **87**, 186 (1963).

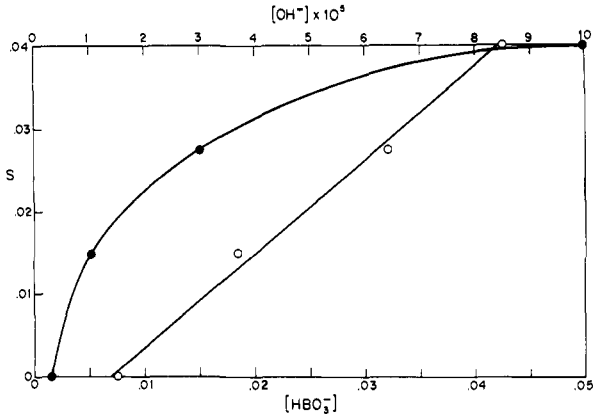

Figure 6. Plots of the slopes of the lines in Figure 5 vs. $[H_2BO_3^-]$ (bottom scale) and $[OH^-]$ (top scale).[26]

an aqueous heme solution directly into monomeric dipyridine hemochrome. Instead a stable intermediate dimeric complex (II) is formed which becomes the exclusive species in solution from 0.006 to 0.1 M pyridine.[21]

These results confilict with those of Gallagher and Elliott,[14] who suggested that a monomeric pyridine hemochrome is formed initially but that this species is converted to another type of monomeric hemochrome upon further additon of pyridine. The structural difference between the two species was, however, never specified. Their assignment was based primarily on matching an experimental spectrophotometric titration with a theoretical curve. Thus, the experimentally observed conversion of aqueous protoheme to the first pyridine hemochrome was found to match the theoretical curve for eq 3.[14] However, such arguments are

$$H_2O\text{–}H_2\text{–}OH_2 + 4P \rightarrow 2P\text{–}H\text{–}P \qquad (3)$$

dangerous since the experimental data also match the theoretical curve for the conversion of I → II in Scheme II where two pyridine ligands are binding to the diaquoheme dimer to form a dipyridine heme dimer. Thus, curve fitting alone cannot be used to make this structural assignment and additional supporting evidence is necessary.

The effect of aqueous micelles on the electronic spectrum of the hemochrome at low pyridine concentrations is, therefore, particularly notable. Both cationic and anionic micelles have been shown to be extremely efficient at molecularly dispersing a variety of porphyrins and metalloporphyrins in aqueous solution.[20] As shown in Figure 3, the λ_{max} of the Soret band of the hemochrome shifts 5 nm to higher wavelength, and its intensity increases dramatically in the presence of cetyltrimethylammonium bromide (CTAB) micelles until it resembles the Soret band of the hexacoordinate dipyridine monomer (III). A virtually identical transformation was induced by anionic sodium dodecylsulfate (SDS) micelles. Such large perturbations cannot be dismissed as due to the change in microenvironment and clearly indicate that a conversion from dimer to monomer is occurring.

The kinetic data obtained at pH 8.5 are completely consistent with these results. Below 0.001 M pyridine, the heme exists largely as a diaquo dimer which oxidizes too rapidly to be observed. As the pyridine concentration is raised, pyridine molecules replace water as axial ligands for the iron and the diaquo dimer is converted to a dipyridine dimer. At 0.006 M pyridine, the conversion is essentially complete.

The oxidation of the dipyridine dimer is, however, more difficult than that of the diaquo dimer because amine ligands are known to raise the midpoint reduction potential of the iron.[22] Some of this stabilization is reflected in an increased activation energy for oxidation, thus retarding the rate to the point where it becomes

Scheme III

$$P\text{-}H_2\text{-}P + buf \underset{}{\overset{K_{eq}}{\rightleftharpoons}} P\text{-}H_2\text{-}buf + P$$

$$P\text{-}H_2\text{-}P \xrightarrow{O_2,\ k_2'} Fe(III) \qquad P\text{-}H_2\text{-}buf \xrightarrow{O_2,\ k_1'} Fe(III)$$

$$k_{obsd} = 2[O_2]\left(\frac{k_1' K_{eq}[buf]}{[P]} + k_2'\right) \qquad (4)$$

detectable. The increase in amplitude of the stopped-flow trace with increasing ligand concentration between 0.001 and 0.006 M, thus, merely reflects the increasing concentration of the dipyridine complex since it is the only reacting species whose oxidation rate is slow enough to be observable. The observed pseudo-first-order rate constant for this oxidation process is, however, completely independent of the ligand concentration over a pyridine concentration range of two orders of magnitude from 0.001 to 0.2 M.

If the pyridine concentration is raised further (above 0.2 M), the dimer begins to break apart. Several different rapidly equilibrating complexes may be present in solution in this pyridine concentration range causing the complications in the kinetics. Since more pyridine is required to dissociate the dimer at higher heme concentrations, the onset of the change in the kinetics shifts to higher pyridine concentrations as the heme concentration is increased.

At a sufficiently high pyridine concentration (>2 M), the heme is completely converted to the hexacoordinate monomeric form and a single process is again observed. However, since this species has two amine ligands per iron instead of one, it oxidizes less readily than the diligated dimer.

It appears clear then that the ligand-induced changes in the kinetics that are observed occur precisely at the points at which the nature of the complexes change as determined by the spectrophotometric titration. Thus, the oxidation process cannot even be detected until two pyridine ligands coordinate to the dimer. Once the dipyridine heme dimer is formed, however, the oxidation rate remains constant until sufficient pyridine is added to break it down.

These results seem to dispute the assumption of Wang and his co-workers in Scheme I that all of the heme in solution is present in the hexacoordinate monomeric form even at a pyridine concentration of 0.1 M.[11] Though their data were obtained in tris buffer and ours in boric acid buffer, it is difficult to understand why a sterically hindered amine like tris($H_2NC(CH_2OH)_3$) would be so much more effective than pyridine at monomerizing heme complexes.

Furthermore, the fact that the oxidation rate of the dipyridine dimer at pH 8.5 is completely independent of the pyridine concentration clearly indicates that dissociation of a pyridine is not a prerequisite for the oxidation process. These results, again, conflict with those obtained earlier by Wang[11] (Scheme I), where a dependence on the reciprocal of the pyridine concentration was observed.

We, therefore, suggest that the inverse order in pyridine observed by Wang in tris buffer could be due to the equilibrium shown in Scheme III, where tris competes with pyridine for a coordination site on the iron of the dimer. The rate law for Scheme III is virtually identical with that obtained for Scheme I.[23] Thus, if both P–H_2–P and P–H_2buf oxidized via outer-sphere processes with $k_1' > k_2'$, a dependence on the reciprocal of the pyridine concentration would also be observed.[24]

This assumption is quite reasonable since it has been shown that strong σ-coordinating ligands enhance the reactivity of iron(II) porphyrins toward oxidizing agents. Thus, the bis(*tert*-butylamine) adduct of iron(II) octaethylporphyrin is more reactive toward molecular oxygen than the bis(pyridine) adduct.[25] Scheme III

(21) See ref 13 where evidence for a monopyridinemonoaquoheme dimer was also observed.

(22) (a) J. H. Wang, *Acc. Chem. Res.*, **3**, 90 (1970); (b) D. G. Davis, *Porphyrins*, **5**, 127 (1978).

(23) The constant 2 has replaced the constant 4 in Scheme I because 2 heme dimers are oxidized by each oxygen.

(24) Even if heme is monomeric in tris buffer under these conditions, the inverse order in pyridine could be explained by an identical scheme using heme monomer instead of dimer.

could, therefore, explain why an inverse order in pyridine is observed in a strongly coordinating buffer such as tris but not in a weakly coordinating buffer such as boric acid.

Support for this interpretation is supplied by the kinetic data in boric acid as a function of pH. As the pH of the medium approaches and crosses the pK_a of boric acid (9.2), the concentration of $H_2BO_3^-$ increases substantially. This species is a better axial ligand than its conjugate acid and it can compete to some extent with pyridine for a coordination site on the iron. Since P-H_2-buf is more reactive toward oxidation than P-H_2-P, an inverse order in pyridine is observed. The magnitude of this inverse-order dependence increases with increasing concentration of the competing ligand causing the slopes of the plots in Figure 5 to increase with pH. The y intercepts of the plots remain constant throughout, however, because the outer-sphere oxidation rate of the dipyridine heme dimer ($k_2' = k_{obsd}/2[O_2] = 7 \times 10^2$ M^{-1} s^{-1}) is pH independent.[26]

All the data, therefore, point toward an outer-sphere oxidation mechanism with no evidence for the intermediacy of any oxyheme complex. These findings are consistent with the general theory that dissociative electron-transfer processes do not occur from low-valence transition metals to π-bonding ligands in low-spin complexes.[26] Instead a peripheral π transfer is the most probable route for this process in analogy with Castro's formulation for monomeric heme complexes in amine solvents.[25] Thus, a loose π complex may be formed between molecular oxygen and the metalloporphyrin followed by electron transfer from the periphery of the ring. Such loose adducts of metalloporphyrins with oxygen as well as other π systems have been previously reported.[27]

Conclusions

(1) Excess pyridine initially converts aqueous protoheme to a dipyridine heme dimer which is stable up to 0.2 M pyridine.

(2) Above this concentration, the dimer is gradually dispersed until at 2 M pyridine, the hexacoordinate dipyridineprotoheme monomer becomes the exclusive species in solution.

(3) The oxidation rate of the dipyridine heme dimer is independent of the pyridine concentration and proceeds entirely via an outer-sphere process.

The mechanism of conversion of the dipyridine heme dimer to the hexacoordinate monomer as well as the oxidation pathway at very high pyridine concentrations is currently still under investigation and will be the subject of a separate publication.

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Reaction of Superoxide Radicals with Copper(II)-Histidine Complexes

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Abstract: A study of the catalytic effect of Cu(II)-histidine complexes upon the disproportionation of superoxide radicals shows that only one complex, $(CuHist_2H)^{3+}$, of six which are known to exist catalyzes the disproportionation in the pH range between 1 and 10. The corresponding second-order rate constant, $k_{7,9} = (3.4 \pm 0.9)10^8$ M^{-1} s^{-1}, is pH independent between 2 and 7. The kinetic results are interpreted by two alternative mechanisms. One is similar to the currently accepted mechanism for superoxide dismutase catalysis; the other is based on the assumption that a transient superoxide complex of $(CuHist_2H)^{3+}$ is formed. The latter assumption is discussed in terms of present knowledge of the structural properties of the copper complexes.

The kinetic and chemical properties of superoxide radicals ($HO_2 \rightleftharpoons O_2^- + H^+$) and their involvement in biological oxidation-reduction reactions have been reviewed in several articles in recent years.[1-5] Research on these species has intensified since the discovery of prevalent superoxide dismutases (SOD), which catalyze their disproportionation to hydrogen peroxide and oxygen.[6]

While at least four metals are known to be involved in various superoxide dismutases, the mammalian enzyme which has a copper atom at the active site, and a zinc atom which is apparently not required for activity, has been most widely studied. It has been suggested that the mechanism of catalytic dismutation of the radicals involves alternate reduction and oxidation of the copper center:[7,8]

$$\text{enzyme-Cu}^{2+} + O_2^- \rightarrow \text{enzyme-Cu}^{+} + O_2 \quad (1)$$

$$\text{enzyme-Cu}^{+} + O_2^- + 2H^+ \rightarrow \text{enzyme-Cu}^{2+} + H_2O_2 \quad (2)$$

where enzyme-Cu represents the enzyme-bound copper ion and the respective rate constants, $k_1 = 2.4 \times 10^9$ and $k_2 = 2.4 \times 10^9$ M^{-1} s^{-1},[8] are near the theoretical limit for diffusion-controlled reactions. The turnover rate is pH independent over the range 4.8-9.5 and declines slightly below pH 4.8. According to the

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