Kinetic Studies on the Incorporation of Iron(II) into Porphyrins[1]

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Abstract: Kinetic measurements were made on the formation of iron(II) hemato-, copro-, and uroporphyrins in 10% pyridine–water solutions at pH 8.2. The rate of the formation of iron(II) uroporphyrin was also measured in water solution at pH 8.2 in which the porphyrin appears to be monomeric even in the absence of pyridine. The rate of incorporation of Fe(II) into the three porphyrins increases as the number of carboxylic acid side chains increases from two for hematoporphyrin, four for coproporphyrin, to eight for uroporphyrin, but the apparent activation energies, 23.7, 23.9, and 24.6 kcal/mole in 10% pyridine–water solution, are equal within experimental error. The rate of incorporation of Fe(II) into uroporphyrin in water solution is considerably faster, with an activation energy of 19.7 kcal/mole. The inhibition of pyridine on the rate of uroporphyrin formation is discussed in terms of ligand strength and the nonenzymatic rate of iron(II) hematoporphyrin formation in water is calculated and discussed.

Recent study of the biosynthesis of the heme proteins, chlorophylls, and vitamin B_{12} has prompted interest in the reaction of the corresponding metal ions with porphyrins. Although several enzyme preparations which catalyze the incorporation of Fe(II) and Co(II) ions into porphyrins have been described, the biochemical mechanism by which the metal ions are incorporated into these cyclic tetrapyrroles is as yet unknown. The present study of the reaction of Fe(II) ion with various porphyrins and factors affecting the rate of reaction of a metal ion with a porphyrin was undertaken with the aim of understanding the enzymatic pathway. Some kinetic studies have been carried out in aqueous detergent solutions, and second-order kinetics have been observed corresponding to a simple bimolecular displacement reaction.[3] Rate constants for the reaction of Fe(II) ion with porphyrins under biological conditions have not been published. It is reported, however, that in nonenzymatic aqueous environments the only ions to show appreciable rates of incorporation into porphyrins are Cu(II) and, to a lesser extent Zn(II), but the biologically important ions (Fe(II), Co(II), Mg(II)) react extremely slowly.[4]

Experimental Section

All spectra were taken with a Cary Model 11 recording spectrophotometer. pH measurements were made with a Beckman Model G pH meter. Kinetic experiments were carried out in either a thermostated water bath or the constant-temperature compartment of the spectrophotometer. Reactions were carried out in glass tubes joined to a 2-cm absorption cell and fitted with removable stopcocks.

Hematoporphyrin dihydrochloride was purchased from Mann Research Laboratory. The dihydrochlorides of coproporphyrin I and uroporphyrin I were prepared from their corresponding esters (Sigma Chemical Co.) by hydrolysis in 20% HCl in the dark followed by evaporation of the resulting solution to dryness *in vacuo*. Neocuproine (2,9-dimethyl-1,10-phenanthroline) and benzylviologen (N,N′-dibenzyl-4,4′-dipyridinium ion) were purchased from Mann Research Laboratory. Sodium dithionite was Baker Chemical Co., technical grade. All other chemicals were reagent grade. Deionized water was used throughout.

Porphyrin stock solutions were prepared by dissolving the porphyrin dihydrochlorides in an equivalent amount of a dilute sodium hydroxide solution (based on $4H^+$ for each hematoporphyrin molecule, $6H^+$ for each coproporphyrin, and $10H^+$ for uroporphyrin) plus acetone (1% by volume of the stock solution). The resulting solutions were diluted to a convenient concentration with 2×10^{-3} *M* Tris buffer (pH 8.2), filtered through paper, and stored frozen in small aliquots. Pyridine solution was prepared by adding concentrated hydrochloric acid to anhydrous pyridine such that a 1:10 dilution with water would give a mixture of pH 8.2. Sodium dithionite solution was prepared by dissolving the solid in Tris buffer, tris(hydroxymethyl)aminomethane, or Tris buffer plus a predetermined amount of dilute sodium hydroxide solution and flushed with oxygen-free nitrogen. The ferrous sulfate solution was also prepared and kept under oxygen-free nitrogen.

Kinetic measurements were all carried out under nitrogen at temperatures kept constant within ±0.2°. The concentrations of porphyrin stock solutions were determined from published extinction coefficients.[5] The concentrations of Fe(II) ion in $FeSO_4$ stock solutions were determined spectrophotometrically after conversion to the *o*-phenanthroline complex. The incorporation of Fe(II) ion into hematoporphyrin in 10% pyridine in water solution was followed by measuring the optical density change at 547 mμ, that into coproporphyrin at 546 mμ and uroporphyrin at 548 mμ. In the absence of pyridine, the incorporation of Fe(II) ion into uroporphyrin in water was followed by measuring the change of optical density at 425 mμ. It was observed that uroporphyrin undergoes rapid photodecomposition under nitrogen atmosphere. Consequently all these solutions were protected from room light with aluminum foil before and during the kinetic measurements.

Following the completion of a reaction, a small amount of $Na_2S_2O_4$ was occasionally injected into the reaction vessel to determine the extent of oxidation. This always amounted to less than 5% for hematoporphyrin. When $Na_2S_2O_4$ was added prior to the addition of $FeSO_4$, no oxidation of the product was observed. Iron(II) uroporphyrin mixtures, in particular, appeared to be much more susceptible to traces of O_2 such that reproducible results could not be obtained in the absence of a small amount of the reducing agent. On the other hand, while $Na_2S_2O_4$ seemed to keep all the added $FeSO_4$ and product heme in the Fe(II) state, it also caused a small but detectable amount of decomposition of the porphyrin prior to the addition of $FeSO_4$ solution. Consequently the initial porphyrin concentration in these solutions was determined from the optical density difference between the free porphyrin and the product heme.

Results

Incorporation of Fe(II) Ion into Hematoporphyrin in 10% Pyridine in Water Solution. Rate measurements

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(3) B. Dempsey, M. B. Lowe, and J. N. Phillips, "Haematin Enzymes," I.U.B. Symposium Series, Vol. 19, J. E. Falk, R. Lemberg, and R. K. Morton, Ed., Pergamon Press Inc., New York, N. Y., 1964, p 29.

(4) J. N. Phillips, "Comprehensive Biochemistry," Vol. 9, M. Florkin and E. H. Stolz, Ed., Elsevier Publishing Co., New York, N. Y., 1963, p 34.

(5) See J. E. Falk, "Porphyrins and Metalloporphyrins," Elsevier Publishing Co., New York, N. Y., 1964, for references to the literature.

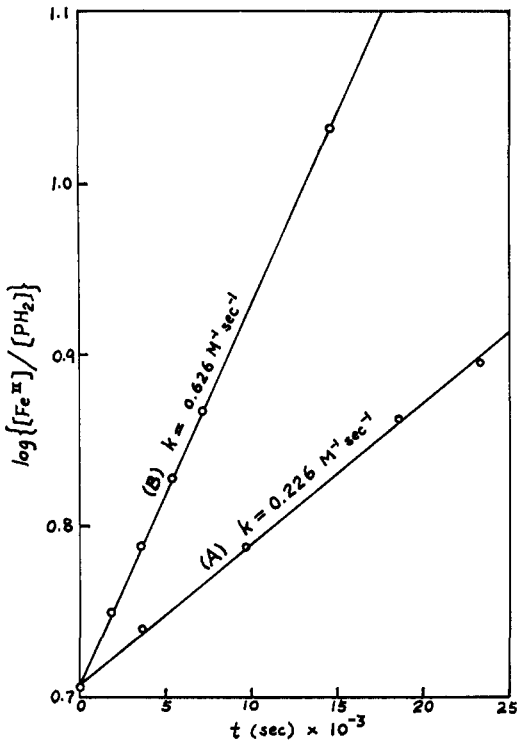

Figure 1. Rate of incorporation of Fe(II) ion into hematoporphyrin in 10% pyridine-water solution at 25°. Initial concentrations: [hematoporphyrin] = 2.00×10^{-5} *M*, [$FeSO_4$] = 1.02×10^{-4} *F*. Curve A, without neocuproine; curve B, with added neocuproine at 1.00×19^{-4} *M*. Both solutions are in 4.0×10^{-4} *M* Tris buffer at pH 8.2.

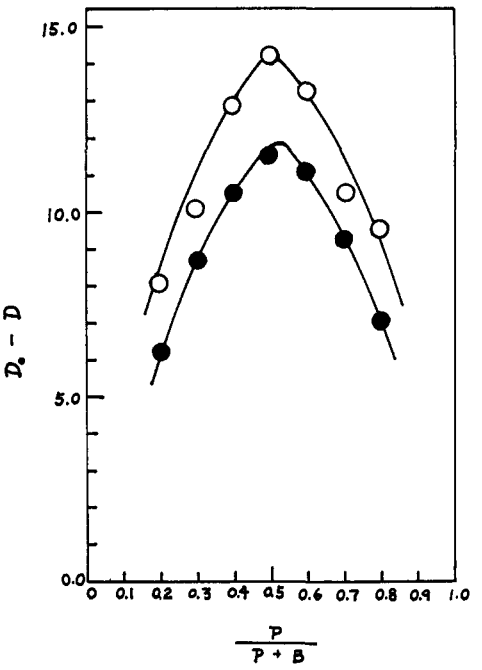

Figure 2. Formation of the hematoporphyrin–benzylviologen complex: O, 0.1 *F* carbonate-bicarbonate buffer at pH 10.2, $D_0 - D$ measured at 375 mμ; ●, 1% pyridine–water solution at pH 7.5, $D_0 - D$ measured at 502 mμ; *D*, optical density (2-cm optical path) of the equilibrium mixture; D_0, optical density of the solution when no complex is formed; P and B, stoichiometric concentrations of hematoporphyrin and benzylviologen, respectively, at constant value of P + B.

were made on the over-all reaction

$$Fe(II) + PH_2 \longrightarrow Fe(II)\text{-}P + 2H^+$$

where PH_2 represents the neutral porphyrin molecule. The measurements were made in 10% by volume pyridine in water solution at pH 8.2, the pH optimum of the enzyme preparations from rat liver.[6] In this solution, hematoporphyrin appears to be monomerically dispersed as demonstrated by the constancy of its molar extinction coefficient (deviation from Beer's law behavior less than 1% in the concentration range 9×10^{-5} to 7.2×10^{-7} *M*). $FeSO_4$ was chosen as the source of Fe(II) ion since it had been used in many enzymatic assays. If the reaction between the Fe(II) ion and the porphyrin PH_2 is represented by

$$Fe(II) + PH_2 \longrightarrow Fe(II)\text{-}P + 2H^+$$

then the integrated second-order rate equation becomes

$$\log \frac{[Fe(II)]}{[PH_2]} = \left(\frac{[Fe(II)]_0 - [PH_2]_0}{2.303}\right)kt - \log \frac{[Fe(II)]_0}{[PH_2]_0}$$

Figure 1 shows two typical rate measurements with the concentration of Fe(II) ion equal to about five times that of the porphyrin. In other measurements the initial ratio $[Fe(II)]_0/[PH_2]_0$ varied from 5 to 1.6. The data give similar linear plots which correspond to the same value of second-order rate constant *k* for the same reacting species and temperature. Similar linear plots were also obtained for the incorporation of Zn(II) ion into hematoporphyrin with $[Zn(II)]_0/[PH_2]_0 = 1$. However in the absence of pyridine the hematoporphyrin molecules seem to aggregate and the reaction system no longer obeys the second-order rate law.

In the course of this work it was discovered accidentally that benzylviologen (N,N′-dibenzyl-4,4′-dipyridinium dichloride) forms molecular complexes with porphyrins. Similar observations have been reported by Mauzerall.[7] By using the method of continuous variation,[8] it was demonstrated that a 1:1 molecular complex was formed between benzylviologen and hematoporphyrin. Some of the results are given in Figure 2. When the viologen is in large excess over the porphyrin, however, a secondary spectrum change occurs, indicating perhaps the formation of higher complexes. Attempts were therefore made to determine an equilibrium constant for the reaction in solutions of hematoporphyrin and viologen at several concentrations for which the porphyrin to viologen ratio was 1:1. In 0.1 *F* carbonate–bicarbonate buffer at pH 10.2 the formation constant of the 1:1 complex was determined at 375 mμ to be $(2.4 \pm 0.5) \times 10^4\ M^{-1}$. The present value seems to be larger than the stability constant of any similar molecular complex reported in the literature. For comparison, the stability constant for the 1:1 complex of methylviologen and ferrocyanide is 52 ± 5,[9] and those for the charge-transfer complexes of the simple pyridinium ions are even smaller.[10]

The above observation suggested the possibility of the catalysis of metalloporphyrin formation by increasing the activation entropy through molecular com-

(6) R. F. Labbe and N. Hubbard, *Biochim. Biophys. Acta*, **41**, 185 (1960); **52**, 130 (1961).

(7) D. Mauzerall, *Biochemistry*, **4**, 1801 (1965).

(8) W. C. Vosburgh and G. P. Cooper, *J. Am. Chem. Soc.*, **63**, 437 (1941).

(9) A. Nakahara and J. H. Wang, *J. Phys. Chem.*, **67**, 496 (1963).

(10) E. M. Kosower, "The Enzymes," Vol. 3, P. D. Boyer, H. Lardy, and K. Myrback, Ed., 1960, Chapter 13.

plex formation between a metal–ligand complex and the porphyrin. Neocuproine (2,9-dimethyl-1,10-phenanthroline) was chosen as the ligand because of its structural similarity to benzylviologen and the steric hindrance of its methyl groups which effectively prevents the formation of 3 to 1 chelate with Fe(II) ion. The steric factor is quite important since 1,10-phenanthroline itself inhibits the incorporation reaction. Table I summarizes the kinetic results on Fe(II)–hematoporphyrin formation at several temperatures with and without neocuproine. A significant catalysis by neocuproine when present at 1:1 ratio with Fe(II) ion was observed. Table II shows that the increase in reaction rate at higher neocuproine concentrations is not linear.

Table I. Second-Order Rate Constants for the Formation of Iron(II) Hematoporphyrin[a]

Temp, °C	[Hematoporphyrin], $M \times 10^5$	[$FeSO_4$], $F \times 10^4$	[Neocuproine], $M \times 10^4$	k, M^{-1} sec^{-1}
25	2.00	1.02	...	0.22
	2.00	1.02	...	0.23
	1.99	1.02	1.00	0.59
	2.00	1.02	1.00	0.63
30	2.00	1.00	...	0.43
	2.00	1.00	1.00	1.25
	1.99	0.960	1.00	1.23
35	1.98	1.02	...	0.83
	2.00	1.02	...	0.85
	1.98	1.02	1.00	2.5
	1.99	1.01	1.00	2.3
40	1.96	1.00	...	1.5
	1.96	1.00	1.00	4.2

[a] 10% by volume pyridine in water solution with 4.0×10^{-4} M Tris buffer at pH 8.2.

Table II. Neocuproine-Catalyzed Iron(II) Hematoporphyrin Formation at 30°[a]

[Neocuproine], M	[$FeSO_4$], M	[Hematoporphyrin], $M \times 10^5$	k, M^{-1} sec^{-1}
5.00×10^{-5}	9.59×10^{-5}	1.97	0.87
1.00×10^{-4}	9.59×10^{-5}	1.99	1.23
1.00×10^{-4}	1.00×10^{-4}	2.00	1.25
5.00×10^{-4}	9.59×10^{-5}	1.99	3.7
1.00×10^{-3}	9.59×10^{-5}	1.97	6.0

[a] 10% by volume pyridine in water solution with 4.0×10^{-4} M Tris buffer at pH 8.2.

The activation energies for both the catalyzed and uncatalyzed reactions are equal to 23.7 ± 1.0 kcal/mole. This is not inconsistent with the hypothesis that the observed catalysis is due to the activation entropy through complex formation. The dependence of reaction rate on the neocuproine concentration could be interpreted in terms of the prior formation of the ternary complex neocuproine–iron(II)–porphyrin, with the Fe(II) sitting above the mean porphyrin plane.[11]

Incorporation of Fe(II) Ion into Coproporphyrin and Uroporphyrin in 100% Pyridine in Water Solution. Preliminary studies on metalloporphyrin formation indicated that the rate of incorporation of transition metal ions into porphyrins is strongly affected by the nature of the previous ligand on the metal ion. In particular an increase in the ligand field strength leads to an increase in the activation energy for metal ion incorporation due to the greater energy required to remove the original ligands in the activation step. Thus pyridine, which was added to the reaction system to keep hematoporphyrin in monomeric form, was found to inhibit the incorporation of Zn(II) ion into the porphyrin. It would therefore be most desirable to avoid the complication and study the rate of Fe(II) ion incorporation into porphyrins which remain in monomeric form even in the absence of pyridine. Unfortunately most porphyrins are polymeric. Only uroporphyrin was reported to be monomeric in its solution in water at pH 8.2.[7]

For comparison, the rate of Fe(II) ion incorporation into uroporphyrin in both water and 10% pyridine in water solution were measured. The data for pyridine in water solution and similar results for coproporphyrin are summarized in Tables III and IV.

Table III. Second-Order Rate Constants for the Formation of Iron(II) Coproporphyrin in 10% Pyridine in Water Solution

Temp, °C	[Coproporphyrin], $M \times 10^5$	[$FeSO_4$], $M \times 10^5$	k, M^{-1} sec^{-1}
25	1.71	3.49	8.2
	1.71	3.46	8.2
30	1.66	3.50	15.6
	1.67	3.53	16.3
35	1.64	3.49	31
	1.64	3.46	29

Table IV. Second-Order Rate Constants for the Formation of Iron(II) Uroporphyrin in 10% Pyridine in Water Solution

Temp, °C	[Uroporphyrin], $M \times 10^5$	[$FeSO_4$], $M \times 10^5$	k, M^{-1} sec^{-1}
25	1.49	2.66	88
	1.45	2.39	80
30	1.50	2.80	1.79×10^2
	1.50	2.73	1.54×10^2
35	1.54	2.78	3.2×10^2
	1.47	2.85	3.3×10^2
	1.44	2.29	3.0×10^2

The activation energies for iron(II) coproporphyrin and iron(II) uroporphyrin formation in 10% pyridine in water solution are 23.9 ± 0.5 and 24.6 ± 1.0 kcal/mole, respectively. Consequently it may be concluded that in these solutions the three porphyrins studied have within experimental error the same activation energy for Fe(II) ion incorporation. Comparison of the data in Tables I, III, and IV shows that the rate constant increases rapidly as the number of carboxylic acid side chains of the porphyrin increases from hematoporphyrin (two) to coproporphyrin (four) to uroporphyrin (eight), a variation which can be attributed entirely to the increase in activation entropy.

Incorporation of Fe(II) Ion into Uroporphyrin in Water Solution. Rate constants for the incorporation of Fe(II) ion into uroporphyrin in water solution at pH 8.2 are summarized in Table V. As may be noticed from Tables IV and V the rate constant in the absence of pyridine is considerably higher and the activation

(11) E. B. Fleischer and J. H. Wang, *J. Am. Chem. Soc.*, 82, 3498 (1960).

energy of 19.7 ± 1.0 kcal/mole is almost 5 kcal smaller than the corresponding quantity in 10% pyridine in water solutions.

Table V. Second-Order Rate Constants for the Formation of Iron(II) Uroporphyrin in Water Solution

Temp, °C	[Uroporphyrin], $M \times 10^5$	[$FeSO_4$], $M \times 10^5$	k, M^{-1} sec^{-1} $\times 10^{-2}$
25	1.40	3.83	8.1
	1.41	2.97	8.5
	1.50	2.62	8.4
	1.42	2.40	8.3
20	1.37	3.83	4.8
15	1.43	2.97	2.8
	1.38	3.83	2.6

Discussion

Previous workers[5] have considered the inductive effect of electron-withdrawing side chains of the porphyrins on the rates of metal ion incorporation. The ease with which various porphyrins were converted to hemes by the liver enzyme followed the same order as the basicity of the porphyrins as reflected by their pK_3 values. Likewise, Phillips[12] found that the rate of Cu(II) ion incorporation into mesoporphyrin was about three times faster than the rate of incorporation into protoporphyrin. These "inductive effects" seem very small when compared to the 370-fold increase in the rate of iron(II) uroporphyrin formation over that for iron(II) hematoporphyrin.

The observed increase in rate constant is most likely electrostatic in origin. If we assume that the first step in the metal ion incorporation reaction is the formation of a complex in which the metal ion sits atop the porphyrin molecule as proposed by Fleischer and Wang[11] and that subsequently this reaction intermediate transforms to the metalloporphyrin, then the larger number of negatively charged carboxyl groups in uroporphyrin would tend to stabilize this sitting-atop complex and hence increase the apparent second-order rate constant. This interpretation is consistent with the observation that in the same solvent system the activation energies, calculated from the present data and summarized in Table VI, are within experimental error equal for all three porphyrins.

Table VI

Porphyrin	Solvent medium	Activation energy, kcal/mole
Hematoporphyrin	10% pyridine in water	23.7 ± 1.0
Coproporphyrin	10% pyridine in water	23.9 ± 0.5
Uroporphyrin	10% pyridine in water	24.6 ± 1.0
Uroporphyrin	Water	19.7 ± 1.0

Finally it may be noticed from Table V that even for the reaction of Fe(II) and uroporphyrin in water solution, the second-order rate constant is two or three orders of magnitude smaller than the corresponding rate constants for the reaction of Fe(II) ion and simpler monodentate ligands. This much slower rate of the present reaction must be due to a higher free-energy barrier at the incorporation step, since the formation of any preincorporation step, if it happens, can only increase the apparent second-order rate constant. In view of our knowledge of the rapid ionic dissociation of the N–H bonds of porphyrins in aqueous solution from H-isotope exchange data,[11] it seems that the only acceptable explanation of the higher free-energy barrier is that the incorporation involves the simultaneous removal of more than one ligand group originally attached to each Fe(II) ion.

(12) M. B. Lowe and J. N. Phillips, unpublished results given on p 35 of ref 5.