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Reactions of Water-Soluble Metalloporphyrins with the Serum Protein, Hemopexin

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Abstract: Rabbit hemopexin is capable of binding a wide variety of synthetic porphyrins and metalloporphyrins including those of the meso-substituted variety. The protein has a requirement for negatively charged peripheral substituents on the porphyrin suggesting that the binding site(s) have a residual positive charge. The stable porphyrin–protein complexes are 1:1 and involve monomeric porphyrin units regardless of the state of aggregation of the porphyrin in solution. The kinetics of the reactions of tetra(4-sulfonatophenyl)porphinatoferrate(III) ($Fe^{III}TPPS$) with rabbit hemopexin has been studied as a function of pH. The protein is capable of interacting with either monomers or dimers leading to substantial changes in metalloporphyrin absorbance and protein fluorescence. When the bound $Fe^{III}TPPS$ is dimeric, a much slower process ensues in which the intermediate complex loses a monomer unit to form the stable product.

Introduction

The degradation of red blood cells in disease-related hemolytic events leads to the occurrence of extraerythrocytic hemoglobin in the bloodstream. Several pathways exist to clear the serum of this circulating hemoglobin (cf. Figure 1). The plasma protein, haptoglobin, binds $\alpha\beta$ dimers of dissociated hemoglobin molecules and transports them to the liver,[2] but even moderate amounts of hemoglobin in the plasma are sufficient to markedly deplete this transfer protein. Part of the remaining hemoglobin dissociates into its components—heme and globin—with conversion of the iron from the 2+ to the 3+ oxidation state.[2] This resultant heme (defined here to be iron protoporphyrin IX) is then complexed by the serum proteins, hemopexin and albumin. Hemopexin, which is present at about $^1/_{50}$ the concentration of albumin, binds the metalloporphyrin about 10^5 times[3,4] more tightly than does the latter protein and carries it to the parenchymal cells of the liver[5-9] where the heme is degraded.[7] In contrast, the heme–albumin complex continues to circulate[6] until apohemopexin again becomes available for heme transport. The metalloporphyrin is then transferred from one protein to the other in a slow step[10] and is carried to the liver for degradation and excretion as a bile pigment.

The absorption spectrum of the heme–hemopexin complex, unlike that of the heme–albumin complex, displays the characteristics of a low-spin hemoprotein; e.g., there is no absorption band near 620 nm.[3,5,11] The low-spin nature of the complex has been confirmed by Mössbauer, ESR, and magnetic circular dichroism (MCD) spectroscopy.[12,13] The visible region MCD spectrum of the ferriprotoporphyrin IX–hemopexin complex closely resembles those characteristic of cytochrome b_5 and other bisimidazole-coordinated ferriprotoporphyrin IX derivatives.[13] Furthermore, while the hemopexin complex of ferrideuteroporphyrin IX exhibits an MCD spectrum similar to that for ferriprotoporphyrin IX, neither the cobalt nor nickel derivatives display these effects.[13]

Although hemopexin interacts with a wide variety of naturally occurring and synthetic metalloporphyrins, only the binding of iron porphyrins induces major changes in the tertiary structure of the protein as evidenced by circular dichroism spectra.[5,14] These changes in tertiary structure may lead to recognition of the complex by hepatocytes.[5,9] Solvent perturbation studies employing ethylene glycol indicate that the heme chromophore is about 70% exposed to solvent when bound to hemopexin.[15] The model which emerges from these data is one in which heme is tightly bonded near or at the surface of he-

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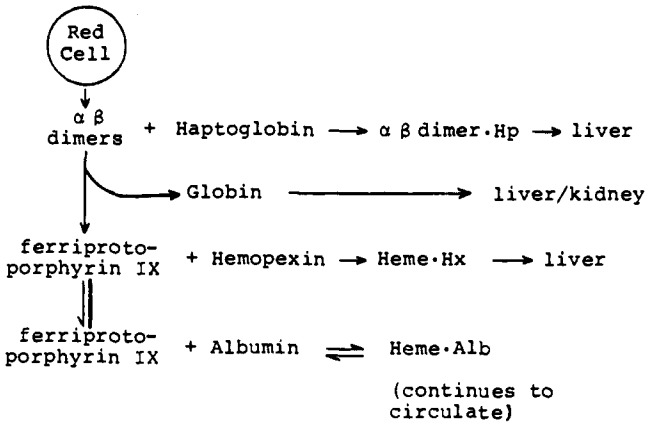

Figure 1. Hemoglobin and heme disposal pathways.

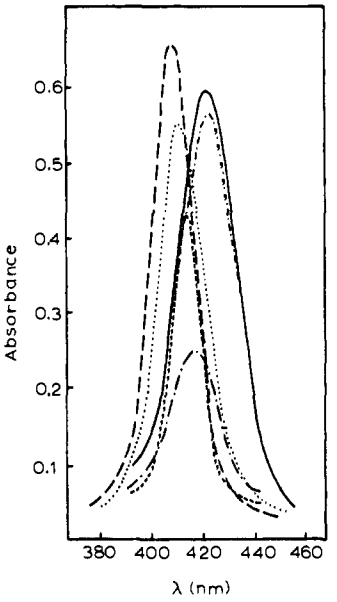

Figure 2. Absorption spectra in the Soret region of copper(II) porphyrins in the absence and in the presence of equimolar rabbit hemopexin, pH 7.4, μ = 0.1 M. CuTCPP (2.2×10^{-6} M): no protein (– – –), with protein (• • •); CuTPPS (1.0×10^{-6} M): no protein (- - -), with protein (- • - • -); CuTMpyP (2.2×10^{-6} M): no protein (—), with protein (- • • • • -).

mopexin with two imidazole rings from histidyl moieties directly attached to the iron(III) site.

The ESR spectra of complexes of both human and rabbit hemopexin with ferriprotoporphyrin IX as well as the absorption spectrum of the deuteroheme-rabbit hemopexin complex are largely unaffected by pH over the range of 6.3–9.3.[14] While these results suggest that there are no major changes in protein conformation over this range, we will present evidence that there are at least minor modifications with pH which are manifested in the kinetic behavior as well as in the fluorescence spectrum of the complex. It is our intention to study the dynamics of the reactions of metalloporphyrins with hemopexin to determine the sequence of events in these interactions. There are advantages to initiating these studies with synthetic water-soluble metalloporphyrins rather than with ferriprotoporphyrin IX itself. Ferriprotoporphyrin IX is known to be extensively dimerized in aqueous solution at all pHs, and some evidence exists for aggregates considerably larger than dimers.[16] In contrast, the extent of tetra(4-sulfonatophenyl)porphinatoferrate(III) ($Fe^{III}TPPS$) dimerization can be controlled by pH and concentration conditions:[17]

$$2FeP(H_2O)_2 \overset{K_D}{\rightleftharpoons} O\text{-}(FeP(H_2O))_2 + 2H^+ + H_2O$$

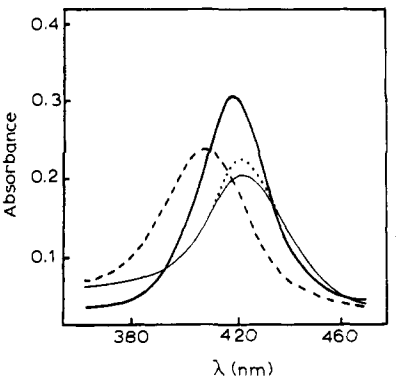

Figure 3. Absorption spectra in the Soret region of iron(III) porphyrins in the absence and in the presence of equimolar rabbit hemopexin, pH 7.4, μ = 0.1 M. FeTPPS (2.2×10^{-6} M): no protein (- - -), with protein (—); FeTMpyP (2.2×10^{-6} M): no protein (• • •), wth protein (—).

with $K_D = 7.9 \times 10^{-9}$ M at 25 °C and μ = 0.1 M. The present report deals primarily with the FeTPPS–rabbit hemopexin system.

Experimental Section

Rabbit hemopexin, purified from rabbit serum as previously described,[11] was prepared at the Scripps Clinic and Research Foundation. Tetrasodium mesotetra-(4-sulfonatophenyl)porphine (Na_4H_2TPPS), mesotetra-(4-carboxyphenyl)porphine (H_6TCPP), and mesotetra-(4-*N*-methylpyridyl)porphine tetraiodide ($H_2TMpyPI_4$) were purchased from Strem Chemicals, Inc., Danvers, Mass. $Fe^{III}TPPS$, $Fe^{III}TMpyP$, $Cu^{II}TCPP$, $Cu^{II}TMpyP$, $Co^{III}TCPP$, and $Co^{III}TMpyP$ were synthesized and purified by literature methods.[17-23] $Cu^{II}TPPS$ was prepared by heating an excess of cupric chloride with Na_4H_2TPPS in distilled water on a steam bath for 1 h. Excess cupric chloride was removed by passing the solution through a cation exchange column. The solution was brought to a pH of 6, and the volume was reduced before acetone was added to precipitate the metalloporphyrin. The product was recrystallized three times from methanol using acetone as a precipitant. Using a combination of spectral and atomic absorption measurements we obtain for $Cu^{II}TPPS$ λ_{max} 412.5 ($\epsilon = 4.16 \times 10^5$ M^{-1} cm^{-1}), 540, 580, and 613 nm. Spectra were determined in water at near zero ionic strength to inhibit aggregation.[24] Other chemicals were reagent grade and were used without further purification.

As aqueous porphyrin and metalloporphyrin solutions are photosensitive, the solutions used in this work were freshly prepared and protected from light. Concentrations were determined using published values of the molar absorptivities.[17-22,24] Protein concentrations were determined from $\epsilon_{280} = 1.10 \times 10^5$ M^{-1} cm^{-1} at pH 7.4, μ = 0.1 M.[25] All experiments were conducted at 25 °C and μ = 0.1 M in NaCl. At pH 6, 6.5, and 7.4, 0.01 M phosphate was used to buffer solutions, whereas at pH 9, a 0.01 M borate buffer was used.

Kinetic experiments were conducted on a Durrum Model D-110 stopped-flow apparatus using both absorbance and fluorescence detection. The latter mode involved excitation at 280 nm and measurement of fluorescent emission using a filter supplied by Durrum which passes radiation of wavelengths 290–330 nm. Absorbance measurements were made on a Cary 14 spectrophotometer, and fluorescence measurements were obtained with a Perkin-Elmer MPF-44A spectrofluorimeter. The absorbance at 280 nm was maintained below 0.08 to minimize inner filter effects, and all values were corrected for dilution and screening effects by control titrations of ovalbumin with CuTPPS and FeTPPS. Circular dichroism measurements were carried out on a Cary Model 61 CD spectropolarimeter at 25 °C. The ellipticity at 231 nm was determined before and after addition of 1 equiv of the metalloporphyrins CuTPPS, FeTPPS, or deuteroheme to the protein at pH 9, 7.5, 6.5, and 6.0. The CD spectra were recorded as previously described;[14] none of these metalloporphyrins has a detectable CD signal in the 350–200-nm region.

Results and Discussion

I. Static Experiments. Spectra in the Soret region were determined for the various porphyrin and metalloporphyrin

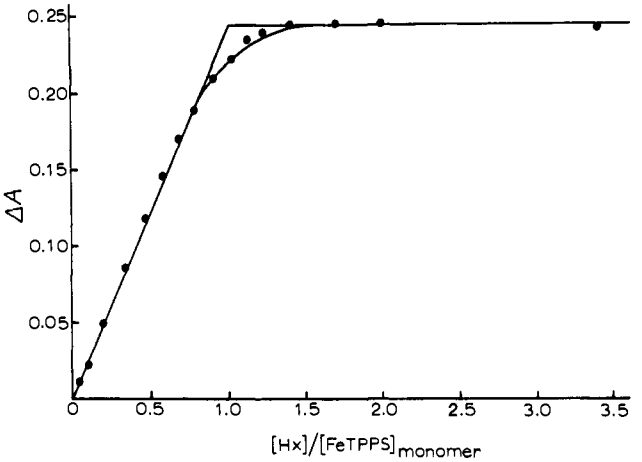

Figure 4. Spectrophotometric titration at 400 nm of rabbit hemopexin (3×10^{-7} to 2×10^{-5} M) with $Fe^{III}TPPS$ at pH 8.9. At this pH, in the absence of protein, the metalloporphyrin is almost totally dimerized at the concentration used ([dimer] = 3.1×10^{-6} M).

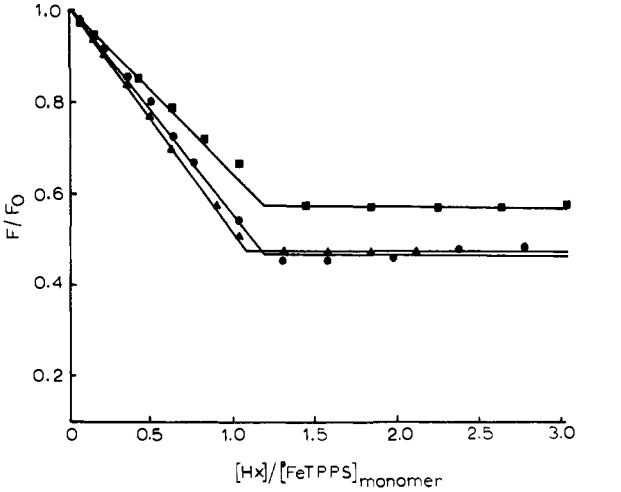

Figure 5. Spectrofluorimetric titrations of rabbit hemopexin (7.27×10^{-7} M, absorbance = 0.07 at 280 nm) with $Fe^{III}TPPS$ at pH 6.5 (▲), pH 7.4 (●), and pH 9.0 (■). The excitation wavelength was 280 nm and the emission wavelength was 335 nm.

species at pH 7.4 both in the absence and presence of equimolar hemopexin. As compared to porphyrins having positive peripheries (TMpyP series), the Soret regions of porphyrins with negatively charged peripheries (TPPS and TCPP series) are markedly influenced by the addition of hemopexin (Figures 2 and 3). The TMpyP series shows only a small decrease in Soret band intensity. For the negatively charged series, the nonmetallo and copper(II) derivatives which have little or no tendency to add axial ligands show a substantial decrease and broadening of their Soret bands (Figure 2), while the iron(III) and cobalt(III) derivatives display a marked red shift in this region (Figure 3).

It has been previously shown[26] that although hemopexin binds deuteroporphyrin and 2,4-disulfonic acid deuteroporphyrin, both of which have negatively charged peripheries, it does not bind the ethylenediamine-substituted protoporphyrin IX, which has positively charged peripheral substituents. Thus, it appears that the region of the binding site(s) of hemopexin for porphyrins has a residual positive charge. The red shifts of the Soret maxima observed for those metalloporphyrins having tendencies to add axial ligands indicate that ligation by one or more protein amino acid residues is occurring[23,27] similar to what is found for heme.[12,13] This conclusion is further substantiated by results obtained in the visible region of the spectrum between 500 and 700 nm. In this spectral region, monomeric $Fe^{III}TPPS$ exhibits absorption bands at 529 nm ($\epsilon = 1.3 \times 10^4$ M^{-1} cm^{-1}) and 648 nm ($\epsilon = 3.8 \times 10^3$ M^{-1} cm^{-1}) with shoulders at 510 and 685 nm. The metalloporphyrin has been shown to involve high-spin iron under the conditions at which the spectrum was determined (pH 3, μ = 0.1 M).[17,28] The bisimidazole complex of FeTPPS, previously shown to involve low-spin iron(III),[28] exhibits a red shift of the Soret band (from 392 to 415 nm) and the appearance of a visible band at 549 nm ($\epsilon = 1.0 \times 10^4$ M^{-1} cm^{-1}). The long-wavelength region of the spectrum now shows shoulders at 610 and 640 nm. The complex between FeTPPS and rabbit hemopexin shows further red shifting of the Soret and major visible bands to 423 nm ($\epsilon = 3.1 \times 10^5$ M^{-1} cm^{-1}) and 568 nm ($\epsilon = 8.7 \times 10^3$ M^{-1} cm^{-1}) and a shoulder at 610 nm. The similarities between the FeTPPS·Hx and the $FeTPPS(Im)_2$ spectra suggest that iron(III) is in a low-spin state in the hemopexin complex bound to two axial ligands, as proposed for the heme·Hx complex.[5,11,13]

Titrations of FeTPPS with hemopexin were conducted using absorbance and fluorescence detection modes. The stepwise addition of hemopexin to FeTPPS at pH 9 produces an absorbance decrease at 400 nm, an increase at 425 nm, and an isosbestic point at 413 nm. At this pH under the concentration conditions used, the FeTPPS is initially greater than 99% dimerized. In Figure 4, $\Delta A = A_0 - A$ is plotted against the ratio of the concentrations of hemopexin to FeTPPS monomer units, where A is the absorbance of the solution at 400 nm in the presence of protein, and A_0 is the absorbance in the absence of protein. The intersection of the two linear portions of the titration curve is at 0.96, suggesting that, as for heme, the stable form of the porphyrin-protein complex involves monomeric FeTPPS with only one strong binding site on the protein. Consistent with this interpretation, the spectrum of the FeTPPS-hemopexin complex remains unchanged from pH 6 to 9, which would alter dimer formation. Further substantiation for this model comes from the results of fluorescence titrations. The ratio F/F_0 in Figure 5 is the relative fluorescence, i.e., the observed fluorescence divided by the initial fluorescence. For the titrations at pH 6.5 and 7.4, the unbound porphyrin is almost totally monomeric, but at pH 9 substantial amounts of dimer are present.[17] Yet all three titration curves are consistent with a 1:1 protein to monomer interaction. It might be noted that the extent of fluorescence quenching at pH 9 is somewhat less than that at pH 6.5 or 7.4, although the final product is the same in all three cases from a stoichiometric point of view. A similar result was found in the titration of CuTPPS at various pHs. This difference in fluorescent quenching may reflect a change in protein conformation between pH 7.4 and 9 which affects tryptophan fluorescence properties.

As may be seen in Figure 5, the binding of FeTPPS follows the stoichiometric binding curve up to the equivalence point preventing a determination of the stability (association) constant for the hemopexin-FeTPPS system. The data from the curved portion of Figure 4 permits a rough estimation of the stability constant for the binding of FeTPPS monomers to hemopexin as $\geq 10^9$ M^{-1}. Thus, it is concluded that the bonding of FeTPPS to hemopexin involves one monomeric porphyrin unit/protein molecule and that the equilibrium constant for the formation of the metalloporphyrin-protein complex is very large and almost certainly greater than 10^9 M^{-1}. These results parallel very closely the ones obtained for the heme-hemopexin system. However, FeTPPS does not emulate heme binding to rabbit hemopexin in all respects. Unlike heme and deuteroheme, neither CuTPPS nor FeTPPS has any influence on the ellipticity of rabbit hemopexin at 231 nm. The enhancement of the ellipticity caused by deuteroheme and heme[14] has been interpreted as arising from a conforma-

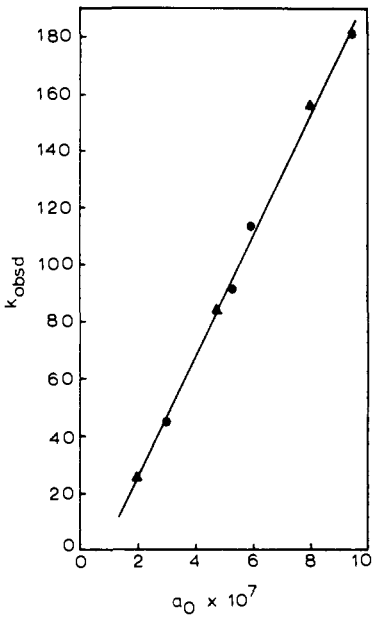

Figure 6. A plot of k_{obsd} vs. the initial concentration of rabbit hemopexin (or Fe^{III}TPPS dimers) at pH 8.9, μ = 0.1 M. The second-order kinetic data were obtained in the absorbance mode at 400 nm (●) or in the fluorescence mode with excitation at 280 nm (▲). The slope of this line is (2.1 ± 0.1) × 10^8 M^{-1} s^{-1}.

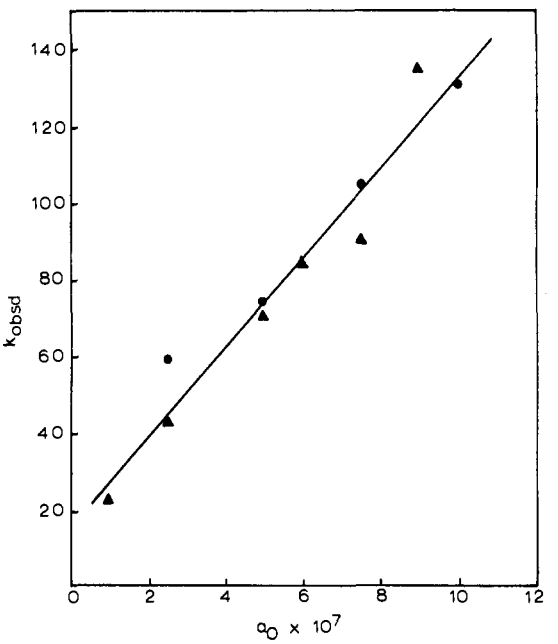

Figure 7. A plot of k_{obsd} vs. the initial concentration of rabbit hemopexin (or Fe^{III}TPPS monomers) at pH 6.5, μ = 0.1 M. The second-order kinetic data were obtained in the absorbance mode at 400 nm (●) or in the fluorescence mode with excitation at 280 nm (▲). The slope of this line is (1.2 ± 0.2) × 10^8 M^{-1} s^{-1}.

tional change of the protein vital to the recognition of the protein–porphyrin complex by hepatocytes.[5,9] Apparently, FeTPPS binding to rabbit hemopexin either does not induce this major modification of the tertiary structure of the protein or interacts with the moieties producing the ellipticity in an different manner.

II. Dynamic Experiments. Kinetic experiments were conducted at pH 6, 6.5, 7.4, and 8.9; μ = 0.1 M both in the fluorescence and absorbance modes. At the concentrations considered here, FeTPPS is extensively dimerized at pH 8.9. The rate constant for conversion of dimer to monomer in the absence of protein was determined by concentration-jump and pH-jump experiments to be 0.13 s^{-1} under these conditions.[28] Because the rate of interaction between the metalloporphyrin and protein is orders of magnitude more rapid than the breakdown of the dimer and because the static experiments indicate one major binding site on the protein, we conducted kinetic experiments with the initial concentration of dimer equal to the concentration of protein. Over the concentration range considered (2×10^{-7} to 1×10^{-6} M), the kinetic profile is monophasic and second order in the quenching of protein fluorescence and in the decrease in absorbance at 393 or 400 nm (reactant peak). However, at 423 nm near the product peak, the reaction is multiphasic. We will return to the 423-nm data.

Figure 6 shows a plot of k_{obsd} vs. the initial concentration of hemopexin (or FeTPPS dimers) at pH 8.9. For the reaction:

$$D + Hx \xrightarrow{k_1} D{\cdot}Hx$$

where D represents the FeTPPS dimer and Hx represents hemopexin, we obtain $k_1 = (2.1 \pm 0.1) \times 10^8$ M^{-1} s^{-1}. The negative intercept reflects experimental difficulties and uncertainties in analyzing second-order data. Both fluorescence and absorbance data at 400 nm correspond to the same straight line, indicating that the quenching of the fluorescence of the protein is concomitant with the interaction with the porphyrin dimer. At pH 6.5, the metalloporphyrin exists as monomeric units, but here too a similar kinetic pattern prevails in the fluorescence and 400-nm absorbance modes. The data are second order for up to 3 half-lives, and a plot of k_{obsd} vs. the concentration of hemopexin (or FeTPPS monomers) is linear (Figure 7) with both fluorescence and absorbance data at 400 nm lying on the same straight line. For the reaction

$$M + Hx \xrightarrow{k_2} M{\cdot}Hx$$

$k_2 = (1.2 \pm 0.2) \times 10^8$ M^{-1} s^{-1}; the intercept again has a high degree of uncertainty, being 16 ± 14 s^{-1}.

Thus, the reaction of FeTPPS with hemopexin involves a very rapid binding step of either porphyrin monomer or dimer which leads to the quenching of tryptophan fluorescence in the protein. That the rate constant is almost twice as large at pH 9 as at pH 6.5 suggests that a pH-dependent conformational change of the protein may occur above pH 6.5 (or 7.4 as estimated earlier) but below pH 9, or that a histidine residue in the binding site may become protonated between pH 6.5 and 9.

The kinetic profile at 423 nm is considerably more complicated at all pH values. At pH 6.5, there is a rapid kinetic effect in the same time range as those observed at 400 nm and in the fluorescence mode, but the effect at 423 nm is biphasic. The coupling is too severe to allow a detailed analysis of the data, but we surmise that at 425 nm we observe a further change in the porphyrin–protein complex which may involve ligation of the metalloporphyrin by the protein and concomitant conformational changes. Thus, at pH 6.5, in the fluorescence and at 400 nm, we see the formation of an intermediate, I, which goes on to form the stable product with a time constant which couples the reaction to the biomolecular step:

$$M + Hx \xrightarrow{k_2 = 1.2 \times 10^8\ M^{-1}\ s^{-1}} I: \quad \text{observed at 400 nm, 423 nm,,and in fluorescence}$$

$$I \xrightarrow{k_3 \sim 10^2\text{-}10^3\ s^{-1}} M{\cdot}Hx: \quad \text{observed only at 423 nm}$$

There are no slower effects discernible at 423 nm at pH 6.5.

At pH 8.9, the kinetic profile at 423 nm is more complicated. Once again, as at pH 6.5, there is a rapid, coupled kinetic effect in about the same time range (several milliseconds) as the ef-

fects observed at 400 nm and in the fluorescence. In addition, there is a much slower process having a half-life in seconds rather than milliseconds. This slow effect is first order for over 3 half-lives and, as mentioned above, has no corresponding change in the fluorescence mode. We propose that this slow process is due to the conversion of the D·Hx complex to the M·Hx product by the release of a monomeric porphyrin unit. To test this model, we performed kinetic experiments over a protein concentration range of 3×10^{-7} to 1×10^{-6} M and at various ratios of hemopexin to porphyrin and observed the following: (1) the slow effect at all ratios in first order; (2) k_{obsd}^{slow} varies linearly as the ratio $[Hx]_0/[D]_0$ up to a value of 1.0, where $[Hx]_0$ and $[D]_0$ are the initial hemopexin and dimer concentrations, respectively. Above that value, there is a break and a new linear dependence on the same ratio appears (Figure 8). Up to a value of 1.0, the following reactions need be considered:

$$D \xrightarrow{k_4} 2M$$

$$DHx \xrightarrow{k_5} MHx + M$$

Appendix I describes the analysis of this model which leads to the equation:

$$k_{obsd}^{slow} = k_4 + (k_5 - k_4)[Hx]_0/[D]_0$$

The data of Figure 8 lead to $k_4 = 0.13\ s^{-1}$ and $k_5 = 0.033\ s^{-1}$.

In the range $1 < [Hx]_0/[D]_0 \leq 2$, the reactions to be considered are:

$$DHx \xrightarrow{k_5} MHx + M$$

$$HxDHx \xrightarrow{k_6} 2MHx$$

leading to (cf. Appendix I):

$$k_{obsd}^{slow} = (2k_5 - k_6) + (k_6 - k_5)\,[Hx]_0/[D]_0$$

From the second linear plot of Figure 8 we obtain that $k_5 = 0.049\ s^{-1}$ and $k_6 = 0.007\ s^{-1}$. The two independent determinations of k_5 give an average value of $\langle k_5 \rangle = 0.041\ s^{-1}$.

Kinetic experiments conducted at lower pH values further substantiate these models for the slow kinetic process. Protein was flowed against porphyrin such that the initial concentration of monomer plus dimer was equal to the initial concentration of hemopexin. At lower pH, the size of the slow effect relative to the fast effect at 423 nm decreases until, at pH 6.5, no slow effect is observed. This is consistent with the decrease in dimer concentration as pH is lowered. In addition, when unbuffered dimer at pH 10 was reacted with a buffered protein solution at pH 6.5, a large slow effect was observed at 423 nm but not seen in the fluorescence.

In summary, we have shown that hemopexin binds a variety of synthetic metalloporphyrins and confirmed the requirement for negatively charged peripheral substituents. The stable porphyrin-protein complexes are 1:1, involving monomeric porphyrin units regardless of the state of aggregation of the porphyrin in solution. The protein is capable of interacting with either monomers or dimers, and this interaction causes a substantial change in the protein fluorescence and metalloporphyrin absorbance. If the bound porphyrin is dimeric, in a much slower step, this intermediate loses a monomer unit to form the stable complex. In the presence of excess protein, there is some evidence that a second protein molecule can bind the second monomeric unit forming an intermediate of the type HxDHx. This requires that, despite the avidity with which hemopexin binds porphyrins, the site of binding must be at or near the surface of the molecule and be sterically nonrestrictive. Indeed, previous studies have shown that heme is about 70% exposed to solvent in the heme-hemopexin complex.[15] This may relate to the mechanism of heme transport to the liver by hemopexin, which has recently been shown to involve heme release and recycling of the protein to the circulation.[9] Work has begun on the dynamics of the heme-hemopexin interaction, and we anticipate reporting on this system in the near future.

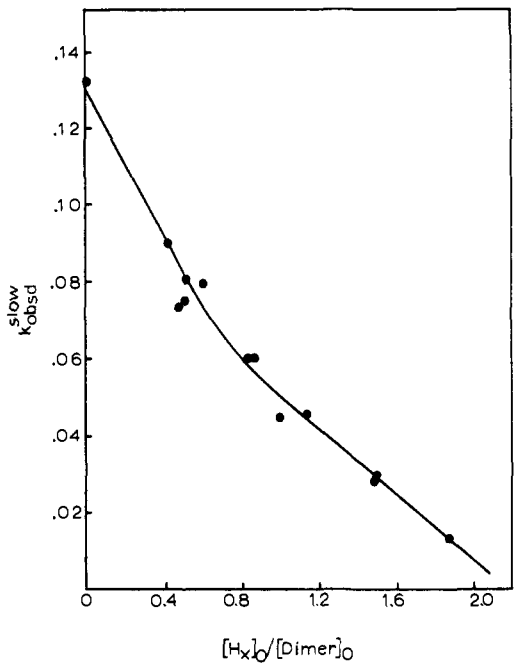

Figure 8. A plot of k_{obsd}^{slow} vs. the ratio of the initial concentration of rabbit hemopexin to the initial concentration of $Fe^{III}TPPS$ dimers. The first-order kinetic data were obtained at pH 8.9, $\mu = 0.1$ M at 423 nm.

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Appendix I

The analysis of the slow effect observed at 423 nm, pH 8.9, begins with a consideration of the possible metalloporphyrin-protein species formed in solution. The dimer is the stable form of the uncombined metalloporphyrin under these conditions, and we consider two intermediate complexes, D·Hx and $D{\cdot}Hx_2$. Employing the result that the binding steps are very rapid, we consider the following:

$$D \xrightarrow{k_4} 2M$$

$$+ Hx \rightleftharpoons K_1$$

$$D{\cdot}Hx \xrightarrow{k_5} MHx + M$$

$$+ Hx \rightleftharpoons K_2$$

$$D{\cdot}Hx_2 \xrightarrow{k_6} 2MHx$$

We neglect the dimerization step

$$M + M \rightarrow D$$

as well as the reaction

$$M + Hx \rightarrow MHx$$

because, under the conditons of these experiments, there is relatively little free monomer or hemopexin present initially.

Part I: Excess Dimer. The reactions to be considered are:

$$D \xrightarrow{k_4} 2M$$

$$D{\cdot}Hx \xrightarrow{k_5} MHx + M$$

We define $[D_T]$ as the total dimer concentration, $[D_T] = [D] + [DHx]$, and since the reaction is observed to be first order, we write:

$$\frac{-d[D_T]}{dt} = k_{obsd}^{slow}[D_T] = \frac{-d[D]}{dt} - \frac{d[DHx]}{dt}$$

$$k_{obsd}^{slow}[D_T] = k_4[D] + k_5[D{\cdot}Hx]$$

which leads to:

$$k_{obsd}^{slow} = \frac{k_4[D] + k_5[D{\cdot}Hx]}{[D_T]}$$

for all time, t. We make use of this conclusion to simplify the expression. First, we define:

$$[D]_i = [D]_0 - [Hx]_0$$

where $[D]_0$ and $[Hx]_0$ are the original concentrations of dimer and protein, respectively. Because the stability constant for the porphyrin-protein complex, K_1, is very large and because a protein molecule binds only one porphyrin molecule, $[D]_i$ represents the initial concentration of free dimer after the binding step but before appreciable conversion to monomer. Similarly, $[DHx]_i = [Hx]_0$. Then, at $t = t_i$:

$$\left(\frac{-d[D_T]}{dt}\right)_i = k_{obsd}^{slow}[D]_0 = k_4([D]_0 - [Hx]_0) + k_5[Hx]_0$$

and

$$k_{obsd}^{slow} = k_4 + (k_5 - k_4)[Hx]_0/[D]_0$$

for all time, t.

Part II: Excess Protein; $1 \leq [Hx]_0/[D]_0 \leq 2$. Now the reactions to be considered are:

$$D{\cdot}Hx \xrightarrow{k_5} MHx + M$$

$$HxDHx \xrightarrow{k_6} 2MHx$$

We are suggesting that in the presence of excess protein, a second molecule of hemopexin can bind to the dimer and that this occurs in a rapid step. The stability constant K_2 for the formation of HxDHx, although large, is assumed smaller than the constant K_1 for the binding of dimer to Hx, and, therefore, little HxDHx is formed when the dimer is in excess of equal to the concentration of Hx.

Reasoning as in part I, we define:

$$[HxDHx]_i = [Hx]_0 - [D]_0$$

$$[DHx]_i = 2[D]_0 - [Hx]_0$$

$$\left(\frac{-d[D_T]}{dt}\right)_i = k_{obsd}^{slow}[D_T]_i$$

where $k_{obsd}^{slow}[D]_0 = k_5(2[D]_0 - [Hx]_0) + k_6([Hx]_0 - [D]_0)$ and $k_{obsd}^{slow} = (2k_5 - k_6) + (k_6 - k_5)[Hx]_0[D]_0$.

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