Hemin Binding to Serum Proteins and the Catalysis of Interprotein Transfer†

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ABSTRACT: The reaction of hemin (Hm) with human hemopexin (Hx) has been studied in a mixed dimethyl sulfoxide (Me_2SO)–water solvent system and in aqueous caffeine solutions. In both media, the kinetics could be described by a single, second-order process:

$$Hm + Hx \xrightarrow{k} Hm\text{–}Hx$$

with $k = 1.8 \times 10^6$ M^{-1} s^{-1} in 40% Me_2SO–water [pH 7.4, $\mu = 0.2$ M (NaCl)] and $k = 3.9 \times 10^7$ M^{-1} s^{-1} in water [pH 7.4, $\mu = 0.2$ M (NaCl), [caffeine] = 0.025 M]. The reaction shows an ionic strength dependence consistent with a residual 1+ to 2+ charge in the vicinity of the binding region of the protein. The kinetics of the transfer of hemin from albumin to hemopexin

$$Hm\text{–}Alb + Hx \xrightarrow{k'} Hm\text{–}Hx + Alb$$

were studied as a function of concentration, ionic strength, pH, and temperature. In experiments conducted at $3 \leq [Alb]_0/[Hx]_0 \leq 20$ where the transfer kinetics are first order, $k' = 5 \times 10^{-3}$ s^{-1} at $\mu = 0.3$ M (NaCl), pH 7.1; the reaction is strongly dependent on ionic strength and choice of electrolyte. The addition of imidazole catalyzes this transfer process via a ligand-mediated pathway with $k' = 5 \times 10^{-3} + 21[Im]_T^2$. At $[Alb]_0/[Hx]_0 = 92$, the noncatalyzed transfer reaction is second order. From the kinetic analysis of the reaction under these conditions, an estimate is made of the distribution of hemin between the two proteins at concentration levels which are characteristic of serum. The association of hemin and hemopexin is approximately 30 times faster than that of hemin and albumin, a finding consistent with the recycling function of hemopexin during heme transport to the liver parenchymal cells.

The degradation of red blood cells in hemolytic events leads to circulation of hemoglobin, and several serum proteins effect hemoglobin clearance. Haptoglobin binds $\alpha\beta$ dimers of dissociated hemoglobin molecules and transports them to the liver (Muller-Eberhard, 1970). Part of the metalloporphyrin is released from hemoglobin not complexed by haptoglobin, and its iron is concomitantly oxidized from the +2 (heme) to the +3 (hemin) oxidation state (Muller-Eberhard, 1978). The latter compound is complexed by the serum proteins hemopexin and albumin. Hemopexin binds the metalloporphyrin 10^5 times more tightly than does albumin (Beaven et al., 1974; Hrkal et al., 1974) and facilitates a receptor-mediated uptake of hemin by the parenchymal cells of the liver (Morgan, 1976; Hershko, 1975; Davies et al., 1979; Muller-Eberhard et al., 1970; Smith & Morgan, 1979). Hemin bound to albumin is gradually transferred to hemopexin (Morgan et al., 1976) which recycles during the heme delivery process (Smith & Morgan, 1979). Thus, the interactions of hemin with hemopexin and the transfer of this metalloporphyrin from albumin to hemopexin are of both chemical and biological interests.

Because of the pronounced tendency of hemin to aggregate in aqueous solutions, our investigations on the reactions of metalloporphyrins with hemopexin were initiated with synthetic water-soluble metalloporphyrins and, in particular, (tetraphenylporphinesulfonato)ferrate(III) (FeTPPS)[1] (Gibbs et al., 1980; Conway & Muller-Eberhard, 1976). The results of these studies can be summarized as follows:

(i) The interaction of synthetic metalloporphyrins with hemopexin, like that with albumin (Parr & Pasternack, 1977), requires negatively charged peripheral substituents. (ii) Hemopexin interacts with both monomers and aggregates, and the interactions cause a substantial change in protein fluorescence and metalloporphyrin absorbance. (iii) Stable complexes involve equimolar monomeric porphyrin units and hemopexin molecules regardless of the state of aggregation of the porphyrin in solution. For FeTPPS, the conversion of the 2:1 metalloporphyrin–protein complex to the stable product is about 3 times slower than the dimer to monomer conversion in the absence of protein:

$$(FeTPPS)_2 + Hx \xrightarrow{k_1} (FeTPPS)_2\text{–}Hx \quad (1)$$

$$(FeTPPS)_2\text{–}Hx \xrightarrow{k_2} FeTPPS\text{–}Hx + FeTPPS \quad (2)$$

with $k_1 = 2.1 \times 10^8$ M^{-1} s^{-1} and $k_2 = 0.041$ s^{-1}.

The kinetics of the binding of hemin to hemopexin to form the 1:1 complex is, in part, the subject of the present paper. Hemin is highly aggregated in aqueous solutions, with the size of the aggregates varying with concentration (Brown et al., 1976, 1970). Therefore, two solvent systems which have been shown to keep this porphyrin monomeric were employed: (i) 40% Me_2SO in water (Collier et al., 1979) and (ii) an aqueous solution of 0.025 M caffeine (Gallagher & Elliott, 1968). We have also studied the transfer of hemin from albumin to hemopexin both with and without added imidazole. Addition of imidazole to a hemin–albumin complex leads to a rapid shift of the Soret band maximum from 405 to 412 nm followed by a further spectral change to 434 nm. The 434-nm absorption maximum is characteristic of aggregated hemin–imidazole complex in aqueous solution (Gallagher & Elliott, 1968;

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[1] Abbreviations: FeTPPS, (tetraphenylporphinesulfonato)ferrate(III); Hm, hemin, the iron(III) derivative of protoporphyrin IX; Hx, hemopexin; Alb, albumin; Im, imidazole; Me_2SO, dimethyl sulfoxide; HSA, human serum albumin.

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Campbell, 1981). The occurrence of the 434-nm band suggests that imidazole competes with albumin for hemin and, thereby, may act as a catalyst in the transfer of hemin from albumin to hemopexin:

$$\text{Hm–Alb} + \text{Hx} + n\text{Im} \rightarrow (\text{Im})_n\text{–Hm–Alb} + \text{Hx} \rightarrow \text{Hm(Im)}_n + \text{Alb} + \text{Hx} \rightarrow \text{HmHx} + \text{Alb} + n\text{Im} \quad (3)$$

In this publication, evidence is presented for catalysis via such a "ligand-mediated pathway".

Materials and Methods

Human hemopexin and albumin were purified from sera (Hrkal & Muller-Eberhard, 1971) at Scripps Clinic and Research Foundation (SCRF). Experiments conducted with fraction V HSA, purchased from Miles Laboratories Inc. Research products, gave results consistent with those obtained with the albumin prepared at SCRF. Recrystallized hemin was obtained from Nutritional Biochemicals Corp., imidazole (Aldrich) was recrystallized from toluene, and dimethyl sulfoxide (Me_2SO) (Aldrich) was vacuum distilled over BaO and stored frozen under nitrogen until use. All other chemicals were reagent grade and used without further purification.

Solution concentrations were determined spectrophotometrically on a Cary 14 UV/vis spectrophotometer. Hemin was dissolved in neat Me_2SO in which $\epsilon = 1.74 \times 10^5$ M^{-1} cm^{-1} at 405 nm (Collier et al., 1979); albumin and hemopexin, dissolved in 0.01 M sodium phosphate buffer, pH 7, were determined from $\epsilon = 3.74 \times 10^4$ M^{-1} cm^{-1} at 279 nm and $\epsilon = 1.10 \times 10^5$ M^{-1} cm^{-1} at 280 nm, respectively (Clark et al., 1962; Seery et al., 1972). Kinetics experiments were conducted on the Cary 14 UV/vis spectrophotometer and a Durrum Model 110 stopped-flow and a Nortech Laboratory stopped-flow apparatus. The latter flow is all glass and was particularly useful for studies involving Me_2SO.

Results

Kinetic Studies in 40% Me_2SO–Water. Me_2SO, a mild denaturing agent (Tandord, 1968), at ≥40% in water (Collier et al., 1979) keeps hemin in a monomeric state. Spectra of hemopexin and the hemin–hemopexin complex in the Me_2SO–water mixture are very similar to those obtained in water. The protein exhibits a λ_{max} 282 nm with a shoulder at 290 nm in both solvent systems, and the hemin–hemopexin complex absorbs at 414 nm in water and 415 nm in the Me_2SO–water mixture. The kinetics of the reaction

$$\text{Hm} + \text{Hx} \xrightarrow{k_3} \text{Hm–Hx} \quad (4)$$

were studied at 25 °C at $\mu = 0.2$ M (NaCl) at a pH of 7.4 (0.01 M sodium phosphate buffer). The initial concentrations of hemin and hemopexin were equal; $[\text{Hm}]_0 = [\text{Hx}]_0$. Plots of $(\text{Abs}_t - \text{Abs}_\infty)^{-1}$ vs. time were linear for over three half-lives with no evidence for additional kinetic processes. A plot of k_3^{exp} ($=k_3[\text{Hx}]_0$) obtained from this simple second-order kinetic data vs. $[\text{Hm}]_0$ is shown in Figure 1. The slope of this line gives $k_3 = (1.76 \pm 0.21) \times 10^6$ M^{-1} s^{-1}. The reaction of hemin with hemopexin was investigated over a temperature range from 5 to 43 °C. The activation energy is 12.6 ± 3.9 kcal/mol and $\Delta S^* = 10.2 \pm 6.5$ eu.

Kinetic Studies in Aqueous Solution with 0.025 M Caffeine. Caffeine effectively disperses hemin aggregates into monomeric units in aqueous solution (Gallagher & Elliott, 1968). Interactions of the π–π and hydrophobic types are involved rather than axial ligation of iron centers (Gallagher & Elliott, 1968; Campbell, 1981). For determination of the optimum caffeine concentration for the present binding studies, both spectral and kinetic criteria were considered. At $[\text{Hm}] = 1.2 \times 10^{-5}$ M, pH 7.4 (0.01 M sodium phosphate buffer), $\mu = 0.2$ M (NaCl), no spectral changes were observed for [caffeine] greater than 8 mM.

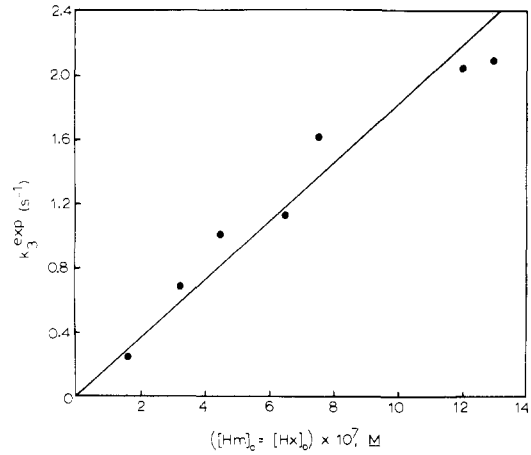

FIGURE 1: Plot of the observed rate constant for the reaction of hemin with hemopexin as a function of initial concentration. The kinetics were studied in a 40% Me_2SO–water solvent system, pH 7.4, $\mu = 0.2$ M, $T = 25$ °C, with $[\text{Hm}]_0 = [\text{Hx}]_0$ in all cases.

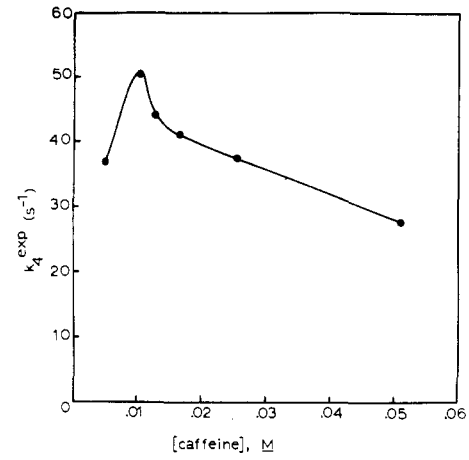

FIGURE 2: Plot of the observed rate constant for the reaction of hemin with hemopexin as a function of caffeine concentration. The kinetics were studied in sodium phosphate buffer, pH 7.4, $\mu = 0.2$ M, $T = 26$ °C.

Under conditions that $[\text{Hx}]_0 = [\text{Hm}]_0$, the observed rate constant (k_4^{exp}) for the reaction

$$\text{Hm} + \text{Hx} \xrightarrow[\text{caffeine}]{k_4^{exp}} \text{Hm–Hx} \quad (5)$$

(where $k_4^{exp} = k_4[\text{Hx}]_0$) shows a caffeine dependence (Figure 2). A caffeine concentration of 0.025 M was chosen for the binding studies because (i) it exceeds the minimum concentratins needed to keep hemin monomeric under the conditions of these experiments and (ii) k_4^{exp} is relatively insensitive to small changes in caffeine concentration above [caffeine] = 0.02 M.

In a series of experiments conducted at 25 °C, pH 7.4, $\mu = 0.2$ M (NaCl), [caffeine] = 0.025, and varying $[\text{Hm}]_0 = [\text{Hx}]_0$, a single second-order kinetic profile was obtained. A plot of k_4^{exp} vs. $[\text{Hm}]_0$ yields a value of $k_4 = (3.89 \pm 0.96) \times 10^7$ M^{-1} s^{-1} (Figure 3). Activation parameters were obtained over the temperature range 14.1–35.4 °C as $E_a = 10.5 \pm 0.70$ kcal/mol and $\Delta S^* = 9.6 \pm 1.2$ eu.

The dependence of k_4 on ionic strength and pH was also determined. A plot of log k_4^{exp} vs. $\mu^{1/2}$ is linear (Figure 4A) with a slope of -1.12 ± 0.37. The reaction rate shows a pH

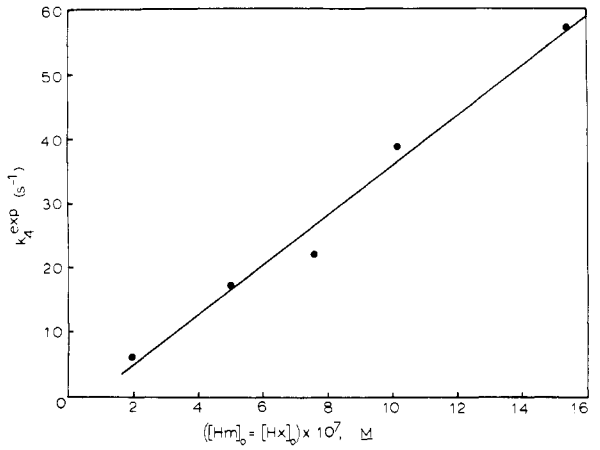

FIGURE 3: Plot of the observed rate constant for the reaction of hemin with hemopexin as a function of initial concentration. The kinetics were studied in water with added caffeine. The conditions were [caffeine] = 0.025 M, pH 7.4, μ = 0.2 M, T = 26 °C, with $[Hm]_0 = [Hx]_0$ in all cases.

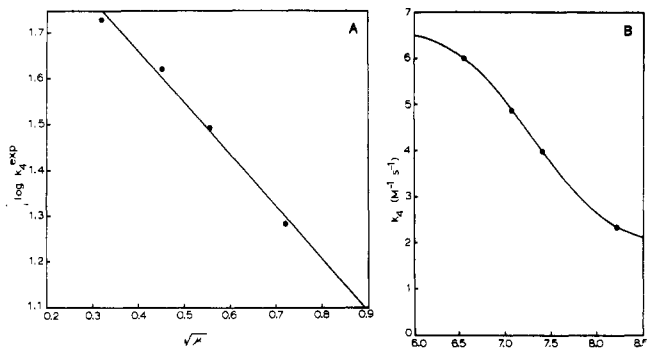

FIGURE 4: (A) Dependence on the observed rate constant for hemin reacting with hemopexin as a function of the square root of the ionic strength. The data were obtained for aqueous solutions; pH 7.4, μ = 0.2 M, T = 26 °C, [caffeine] = 0.025 M. (B) Dependence of the rate constant for hemin reacting with hemopexin on pH. The data were obtained for aqueous solutions; [caffeine] = 0.025 M, μ = 0.2 M, T = 26 °C.

dependence as well; the theoretical curve of Figure 4B was obtained from an equation of the form

$$k_4 = (k_{0,4}[H^+] + k'_{0,4}K_a)/([H^+] + K_a) \quad (6)$$

where K_a is the acid dissociation constant for an ionizable group on the protein, $k_{0,4}$ is the rate constant for hemin with the protonated form of the protein, and $k'_{0,4}$ is the rate constant for the deprotonated form. From these results pK_a = 7.25, $k_{0,4} = 6.68 \times 10^7$ M^{-1} s^{-1}, and $k'_{0,4} = 1.81 \times 10^7$ M^{-1} s^{-1}.

Hemin Transfer Studies (No Imidazole Added). The kinetics of the reaction

$$\text{Hm–Alb} + \text{Hx} \xrightarrow{k_5} \text{Hm–Hx} + \text{Alb} \quad (7)$$

were studied as a function of concentration, ionic strength, pH, and temperature. In all experiments, the serum albumin concentration was kept in excess of the hemin concentration to minimize binding of the metalloporphyrin at other than the primary site (Parr & Pasternack, 1977). The kinetics were studied at 414 nm which is the absorption maximum of the Hm–Hx complex. The reaction is *first order* for several half-lives with $k_5 = 5 \times 10^{-3}$ s^{-1} at 24 °C, μ = 0.3 M (NaCl), pH 7.05, in the range $3 \leq [Alb]_0/[Hx]_0 \leq 20$. Consistent with this finding, initial rate studies conducted in this range of protein ratios showed the reaction to be first order in the hemin–albumin complex but zero order in hemopexin and free albumin. At higher $[Alb]_0/[Hx]_0$ ratios, the kinetic data

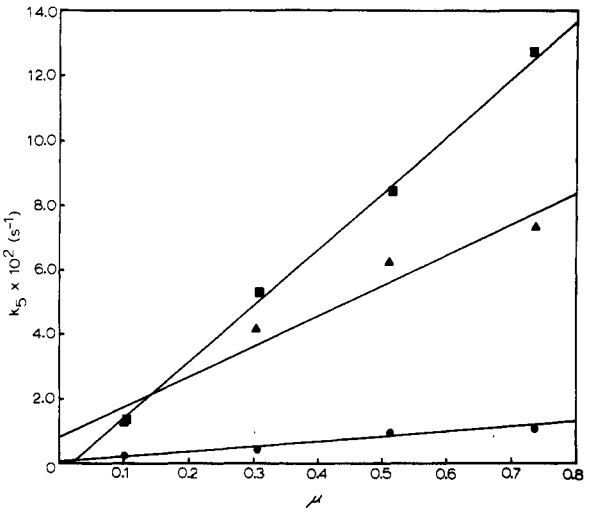

FIGURE 5: Dependence of the rate constant for transfer of hemin from serum albumin to hemopexin as a functon of ionic strength: (■) $LiNO_3$, (▲) $NaNO_3$, and (●) NaCl. The conditions were pH 7.05, T = 24 °C, and $[Alb]_0/[Hx]_0$ = 5. The slopes of the lines are 1.5 $\times 10^{-2}$ M^{-1} s^{-1} for NaCl, 9.4 $\times 10^{-2}$ M^{-1} s^{-1} for $NaNO_3$, and 15 $\times 10^{-2}$ M^{-1} s^{-1} for $LiNO_3$.

Table I: pH Dependence of the Transfer of Hemin from Albumin to Hemopexin

(A) uncatalyzed pathway (μ = 0.30 M, T = 24 °C)		(B) $[Im]_T = 4.0 \times 10^{-2}$ M (μ = 0.30 M, T = 24 °C)	
pH	k (s^{-1}) $\times 10^3$	pH	k_{cat} (s^{-1}) $\times 10^2$
6.30	66	6.81	74
6.50	63	7.05	39
6.70	50	7.13	32
6.97	5.9	7.19	26
7.15	5.0	7.23	39
7.35	5.3	7.24	34
7.38	5.4	7.32	28
7.40	5.9	7.36	34
		7.57	32
		7.75	24

cannot be analyzed as either first or second order, but at $[Hm]_0 = [Hx]_0 = 5.70 \times 10^{-6}$ M and $[Alb]_0 = 5.25 \times 10^{-4}$ M [i.e., $[Alb]_0/[Hx]_0 = 92$, μ = 0.3 (NaCl), pH 7.05, T = 24 °C], the kinetic profile is simple *second order* with k_5^{exp} ($=k_5[Hx]_0$) $= 1.78 \times 10^{-3}$ s^{-1}.

In experiments conducted at $3 \leq [Alb]_0/[Hx]_0 \leq 20$, where the transfer kinetics are first order, the rate of the reaction is strongly dependent on ionic strength. A plot of k_5 vs. μ is shown for several electrolytes in Figure 5; in each case there is a linear dependence of k_5 on ionic strength. The value of the slope depends on the nature of the electrolyte with the anion showing a greater influence than the cation. The reaction rate approximates zero in the limit of zero ionic strength. In subsequent experiments, sodium chloride was used to maintain a constant ionic strength of 0.3 M.

The rate of transfer was also examined as a function of pH and temperature. The reaction is about 10 times faster below pH 7 than at neutral and basic pH. In the pH range 7.0–7.7, virtually no pH dependence of the hemin transfer exists (cf. Table I, part A). The activation parameters for the transfer were obtained at pH 7.05 in a study in which the temperature ranged from 10 to 37 °C as E_a = 4.6 kcal/mol and ΔS^* = −56.0 eu.

Hemin Transfer Studies (Imidazole Added). Vacuum dialysis experiments were conducted with Millipore CX-10 membranes to test the hypothesis based on spectral evidence that imidazole can remove hemin from albumin. Hemin does

Table II: Dialysis Experiments[a]

contents of vessel	membrane	abs[b] (%)
hemin, 0.3 M NaCl	colored (brown)	30
hemin, 0.3 M NaCl, 0.1 M imidazole	colored (red)	32
hemin-Alb, 0.3 M NaCl	unaffected	≃2
hemin-Alb, 1.0 M NaCl	unaffected	≃2
hemin-Alb, 0.3 M NaCl, 0.1 M imidazole	colored (red)	37
hemin-Alb, 0.1 M imidazole	colored (red)	35
hemin-Hx, 0.3 M NaCl	unaffected	0
hemin-Hx, 0.3 M NaCl, 0.1 M imidazole	unaffected	0

[a] [Hemin] = 1.0×10^{-5} M, pH 7.0. [b] Percent absorbance = (decrease in absorbance after replacement of 50% of volume × 100)/original absorbance.

not pass through the membrane but is strongly adsorbed to the surface and is effectively removed from the solution to a theoretical extent (39% depletion when half the volume has been replaced). As may be seen from Table II, when the hemin is bound to albumin or hemopexin, no hemin becomes adsorbed to the surface membrane. When imidazole, in concentrations ranging from 0.1 to 1 M, is added to the hemin–hemopexin complex, still no hemin is removed from solution. However, imidazole effectively removes hemin from albumin by forming a complex which is adsorbed to the membrane. Thus, the dialysis experiments confirmed the suggestion derived from spectral evidence (vide supra) that imidazole can compete with albumin but not with hemopexin for hemin. Kinetic experiments were attempted to investigate the reaction of imidazole with hemin–albumin. Measurement of the development of the 412-nm band requires the stopped-flow technique in the 0.1–1 M imidazole range, pH 7.05, 25 °C, μ = 0.3 M. The reaction is biphasic and shows an imidazole dependence. The analysis of the kinetic data is further complicated by the formation of an additional species which absorbs at 434 nm. This latter process is also biphasic and shows a higher order dependence on imidazole than does the faster process. The overall result of these events indicates that the two sequential processes (the formation of the 412-nm and the 434-nm species) are extremely complicated. However, it can be surmised from these experiments that the initial interaction of imidazole with the hemin–albumin complex is far more rapid than the transfer of hemin from albumin to hemopexin. Therefore, for simplification of the analysis of the transfer reaction, kinetic experiments were conducted by simultaneous addition of imidazole and hemopexin to a solution of hemin–albumin. The transfer kinetics prove to be first order, and the rate constant, k_{cat}, was determined as a function of total imidazole concentration, pH 7.05, μ = 0.3 (NaCl), 25 °C, as shown in Figure 6.

$$k_{cat} = 4.7 \times 10^{-3} + 21[\mathrm{Im}]_T^2 \quad (8)$$

The imidazole concentration range was not extended beyond 0.5 M to avoid the severe coupling reactions during the formation of the 434-nm species which interfere with the transfer kinetics. It should be noted that the transfer rate constant, for the uncatalyzed process obtained as an extrapolation of the results of these experiments, is in excellent agreement with the value obtained from solutions to which no imidazole was added. Experiments conducted at $[\mathrm{Im}]_T = 4.0 \times 10^{-2}$ M as a function of pH showed that there is no appreciable pH dependences in k_{cat} between pH 7 and 7.8 (Table I, part B).

Discussion

Previous work has shown that the interactions of hemin with the serum proteins hemopexin and albumin differ in several important respects including the avidity of binding and the spin state of the iron(III) center. The stability constant for binding of hemin by hemopexin is estimated to be 5 orders of magnitude greater than that for binding by albumin (Beaven et al., 1974; Hrkal et al., 1974). The hemin–albumin complex contains a high-spin five-coordinate iron(III) center (Beaven et al., 1974; Parr & Pasternack, 1977), but in the hemin–hemopexin complex, iron is low spin and six-coordinate (Morgan, 1976; Hrkal & Muller-Eberhard, 1971; Bearden et al., 1974; Morgan & Vickery, 1978). The present work on the kinetics of the hemin–protein interactions reveals further differences between these two proteins.

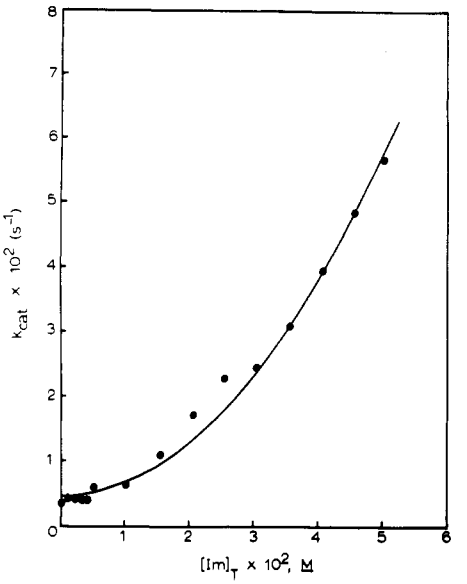

FIGURE 6: Dependence of the rate constant for transfer of hemin from serum albumin to hemopexin as a function of added imidazole. The conditions were pH 7.05, μ = 0.3 M (NaCl), T = 24 °C, and $[\mathrm{Alb}]_0/[\mathrm{Hx}]_0 = 4$.

In 40% Me_2SO–water, the reaction of hemin with hemopexin proceeds without any slow conformational change as shown in eq 4. In the same solvent medium, the reaction with albumin involves a slow conformational change to form the final complex (Adams & Berman, 1980):

$$\mathrm{Hm} + \mathrm{Alb} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \mathrm{I} \qquad \mathrm{I} \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} \mathrm{Hm\text{–}Alb} \quad (9)$$

with $k_1' = 1.7 \times 10^5$ M^{-1} s^{-1}, $k_{-1}' = 10$ s^{-1}, $k_2' = 6.3$ s^{-1}, and $k_{-2}' \simeq 9 \times 10^{-4}$ s^{-1}. The activation parameters for the *binding step* are quite similar for the two proteins: E_a = 12.5 kcal/mol and $\Delta S^\ddagger$ = 5.5 eu for albumin and 12.6 kcal/mol and 10.2 eu for hemopexin.

Similar kinetic patterns are observed in aqueous solution in the presence of caffeine. The reaction of hemin with hemopexin yields a single second-order reaction profile with a rate constant of 3.9×10^7 M^{-1} s^{-1}, about 20-fold greater than that obtained in 40% Me_2SO–water. The value of $\Delta S^\ddagger$ is similar in the two media, but the activation energy barrier is some 2 kcal lower in the aqueous system. The reaction of hemin with albumin was also examined in aqueous medium in the presence of caffeine but the color change is small and the reaction, like that in 40% Me_2SO–water, is biphasic. Therefore, we could only estimate the rate constant for the bimolecular step as being $\simeq 7 \times 10^5$ M^{-1} s^{-1}.

The fact that in both solvent systems studied the rate constant for the bimolecular step is larger for hemopexin than for albumin may have important biological implications. The serum concentration of hemopexin is only $^1/_{70}$th the concen-

tration of albumin. If the two proteins would react at equal rates with free hemin, at low hemin concentrations ≃99% of the metalloporphyrin would be bound by the albumin, which keeps hemin in the circulation rather than bound to hemopexin which facilitates hemin uptake by the liver. From the present binding studies, we estimate that hemopexin binds hemin from 10 times (in 40% Me_2SO) to about 50 times (in water) faster than does albumin. These findings suggest that the distribution of hemin between these two proteins in the bloodstream is shifted in favor of hemopexin to an extent far more efficacious than previously thought (Morgan et al., 1976). We will return to this point during the discussion of the transfer kinetics given below.

Previous studies have shown that hemopexin binds negatively but not positively charged porphyrins (Gibbs et al., 1980; Conway & Muller-Eberhard, 1976). Consistent with this fact, the dependence of the binding rate constant on ionic strength indicates that the binding site of hemopexin has a residual positive charge. We have shown previously that the "effective" charge type of metalloporphyrins for Debye–Hückel type calculations is roughly half the formal charge type (Pasternack et al., 1975), suggesting that the residual positive charge at the binding site of hemopexin is about 1+ to 2+. Our results suggest that there is an ionizable acid group in this region of the molecule having a pK of 7.25 which contributes to this positive charge. When this moiety is protonated, the rate constant for binding is some 3.7 times larger than when it is deprotonated.

As a detailed picture of the kinetics of the binding of hemin to serum proteins emerges, it appears worthwhile to discuss some general features of the interaction. Although the reaction of monomeric hemin with hemopexin involves several steps including the axial ligation of imidazole side chains of histidyl moieties (Bearden et al., 1974; Morgan & Vickery, 1978), all steps subsequent to the bimolecular step are sufficiently rapid so that the kinetics are almost deceptively simple. This can be contrasted with the more complicated kinetics for the interaction of hemin with albumin, a protein which has more general biologial binding functions. Furthermore, although hemopexin binds meso-substituted porphyrins, it accommodates them less easily than it does hemin. The bimolecular rate constant for hemopexin with O-$(FeTPPS)_2^{8-}$ is large, but the conversion of this dimer–protein complex to the final products is quite slow. When the hemin–hemopexin interaction in aqueous solution without added caffeine was attempted, the kinetic data could not be completely analyzed because of the multiplicity of hemin forms in solution. Yet, it is certain that there are no major slow processes associated with the hemin reaction. Therefore, when hemin aggregates bind to hemopexin, the conversion to the final monomeric hemin–hemopexin product is catalyzed by the protein (Brown et al., 1970; Pasternack et al., 1973); in contrast the protein inhibits the monomerization of O-$(FeTPPS)_2^{8-}$.

It has been suggested that the binding of hemin by hemopexin is best understood in terms of an induced-fit model (Kodicek et al., 1981; Koshland & Neet, 1968). The binding of meso-substituted metalloporphyrins may not induce this conformational change. No enhancement of the protein 231-nm band in circular dichroic spectra, characteristic for the hemin–hemopexin interaction, is observed for the interaction of FeTPPS and hemopexin (Gibbs et al., 1980). FeTPPS may not enter the heme binding site of Hx to an extent sufficient to bring its active center into the area needed for monomerization catalysis.

The transfer of hemin from serum albumin to hemopexin can be interpreted in terms of a two-step mechanism:

$$\begin{aligned} \text{Hm–Alb} &\underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} \text{Hm + Alb} \\ \text{Hm + Hx} &\xrightarrow{k_0'} \text{Hm–Hx} \end{aligned} \tag{10}$$

Application of the steady-state approximation to free hemin leads to the expression

$$\text{rate} = \frac{k_0[\text{Hm–Alb}]}{1 + k_{-0}[\text{Alb}]/(k_0'[\text{Hx}])} \tag{11}$$

In the limit that $1 \gg k_{-0}[\text{Alb}]/(k_0'[\text{Hx}])$ ($[\text{Alb}]_0/[\text{Hx}]_0 \leq 20$), the kinetics appear first order for three half-lives with $k_0 = 5.5 \times 10^{-3}$ s^{-1} at pH 7.05, $\mu = 0.30$ M, 24 °C (cf. Table I). The activation parameters determined here, $E_a = 4.6$ kcal/mol and $\Delta S^{\ddagger} = -56$ eu, are similar to those obtained in 40% Me_2SO–water for the conversion of I, the intermediate in eq 9, to the final hemin–albumin complex (Adams & Berman, 1980). This process has been suggested as involving a rearrangement of the intially formed hemin–albumin complex to one in which the hemin gains access to a specific binding site in the interior of the protein. In the process studied here, the rate-determining step involves hemin leaving its binding site and moving toward the surface of the protein molecule from which it dissociates. This is followed by a rapid reaction of hemopexin with the hemin in solution.

As $[\text{Alb}]_0/[\text{Hx}]_0$ increases, the limiting case discussed above is no longer applicable until at $[\text{Alb}]_0/[\text{Hx}]_0 \geq 92$ the second limiting case $[1 \ll k_{-0}[\text{Alb}]/(k_0'[\text{Hx}])]$ is reached and

$$\text{rate} = \frac{k_0k_0'[\text{Hm–Alb}][\text{Hx}]}{k_{-0}[\text{Alb}]_0} \tag{12}$$

At this protein ratio, the kinetics are determined as being second order and (cf. eq 7) $k_5 = k_0k_0'/(k_{-0}[\text{Alb}]_0)$. From $k_0 = 5.5 \times 10^{-3}$ s^{-1} and k_5 ($=k_5^{exp}/[\text{Hm}]_0$) $= 3.12 \times 10^2$ M^{-1} s^{-1} at $[\text{Alb}]_0 = 5.25 \times 10^{-4}$ M, we obtain that $k_0'/k_{-0} = 30$. We have thus determined in a totally independent manner that the rate constant for the binding of hemin by hemopexin in aqueous solution is about 30 times greater than that for the binding to albumin.

There is a marked dependence of the transfer kinetics on electrolyte concentration and composition. Part of this effect may be due to the influence the ionic strength has on the pK_a of the protein (Voet et al., 1981). However, two pieces of experimental evidence suggest that electrolytes have a more profound effect: (i) the transfer kinetics have a stronger dependence on μ than could be predicted from the Debye–Hückel theory, and (ii) the dependence is a specific rather than a general one. The catalytic efficiency of these electrolytes is weak (it would take 140 M $LiNO_3$ to be as effective a catalyst as 1 M imidazole, vide infra). Carbonate and phosphate anions, which are found in the bloodstream in appreciable concentrations, may also exert a catalytic action on the heme transfer process. These investigations are in progress.

Addition of imidazole to the reaction medium significantly enhances the rate of transfer from albumin to hemopexin. That two imidazole molecules are involved (cf. eq 8) suggests the rapid formation of $Hm(Im)_2$ in the albumin pocket which disrupts the hemin–albumin interaction and enhances the rate-determining conformational change of albumin. Subsequently, $Hm(Im)_2$ floats free in solution where it is scavenged by hemopexin before it can aggregate. Small molecules in the bloodstream (dipeptides, oligopeptides) containing histidyl moieties may well be capable of catalyzing the hemin transfer

in a like manner, and this possibility is presently being considered.

Registry No. Hm, 16009-13-5; lithium, 7439-93-2; sodium, 7440-23-5; nitrate, 14797-55-8; chloride, 16887-00-6; Im, 288-32-4.

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Complete Amino Acid Sequence of the Catalytic Chain of Human Complement Subcomponent $C\bar{1}r$[†]

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ABSTRACT: The amino acid sequence of human $C\bar{1}r$ b chain has been determined, from sequence analysis performed on fragments obtained by CNBr cleavage, dilute acid hydrolysis, tryptic cleavage of the succinylated protein, and subcleavages by staphylococcal protease. The polypeptide chain contains 242 amino acids (M_r 27 096), and the sequence shows strong homology with other mammalian serine proteases. The histidine, aspartic acid, and serine residues of the active site (His-57, Asp-102, and Ser-195 in bovine chymotrypsinogen) are located at positions 39, 94, and 191, respectively. The chain, which lacks the "histidine-loop" disulfide bridge, contains five half-cystine residues, of which four (positions 157–176 and 187–217) are homologous to residues involved in disulfide bonds generally conserved in serine proteases, whereas the half-cystine residue at position 114 is likely to be involved in the single disulfide bridge connecting the catalytic b chain to the N-terminal a chain. Two carbohydrate moieties are attached to the polypeptide chain, both via asparagine residues at positions 51 and 118.

The first component of human complement classical pathway, C1, is a calcium-dependent complex consisting of glycoproteins C1q, C1r, and C1s.[1] C1q is considered as a recognition unit, whereas C1r and C1s, which are zymogens of proteases, are sequentially activated through limited proteolysis during C1 activation. The zymogen C1r is autocatalytically activated to its protease form $C\bar{1}r$ (EC 3.4.21.41), which in turn mediates C1s activation [see review by Sim (1981a)]. The active

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[1] Abbreviations: iPr_2P-F, diisopropyl phosphorofluoridate; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-(L-tosylamido)butan-2-one; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid. The nomenclature of complement components is that recommended by the World Health Organization (1968); activated components are indicated by a superscript bar, e.g., $C\bar{1}r$.

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