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Kinetics of Hemoprotein Reduction and Interprotein Heme Transfer†

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ABSTRACT: The transfer of hemin from one protein to another is an event biologically important for the conservation of heme iron. Hemin entering the circulation (or added to serum) is mainly bound by albumin and then transferred to hemopexin [Morgan, W. T., Liem, H. H., Sutor, R. P., & Muller-Eberhard, U. (1976) *Biochim. Biophys. Acta 444*, 435–445], and we are now investigating which mechanisms may be operative in enhancing this process. The presence of imidazole has been demonstrated to accelerate hemin transfer from albumin to hemopexin [Pasternack, R. F., Gibbs, E. J., Hoeflin, E., Kosar, W. P., Kubera, G., Skowronek, C. A., Wong, N. M., & Muller-Eberhard, U. (1983) *Biochemistry 22*, 1753–1758]. The present work is an examination of the effect of the reduction of albumin-bound hemin on the rate of its transfer to hemopexin. Hemin (Hm^{III}; ferriprotoporphyrin IX) was reduced to Hm^{II} (ferroprotoporphyrin IX) by the addition of sodium dithionite under argon. The reduction kinetics of Hm^{III} to Hm^{II} were studied separately in the two complexes: with human serum albumin (HSA), which binds up to 20 mol of heme/mol (the first mole with $K \simeq 10^8$), and with hemopexin (HHx), which binds heme equimolarly ($K \simeq 10^{13}$). The rate of reduction of Hm^{III} to Hm^{II} on HSA was first order over several half-lives and linearly dependent on $[S_2O_4^{2-}]^{1/2}$. At $[HSA]_0/[Hm^{III}] = 3$, the k_{obsd} was $(5 \times 10^{-3}) + 0.75[S_2O_4^{2-}]^{1/2}$, and with $[HSA]/[Hm^{III}] \sim 25$, the k_{obsd} was $(2 \times 10^{-3}) + 0.25[S_2O_4^{2-}]^{1/2}$. The reduction of Hm^{III} to Hm^{II} on human hemopexin (HHx) is much more rapid with $k_{obsd} = (2.5 \times 10^3)[S_2O_4^{2-}]^{1/2}$. The transfer of Hm^{II} from HSA to HHx was studied by adding dithionite to Hm^{III}·HSA and mixing this with HHx. The transfer was biphasic, consisting of two first-order processes, k_f and k_s, independent of $[Hm^{III}\cdot HSA]_0$, $[HSA]_0$, and $[HHx]_0$, but with a slight dependence on pH and ionic strength. The transfer of Hm^{II} from HSA involves two steps that may be due to HSA existing as two noninterconverting conformers [Moehring, G. A., Chu, A. H., Kurlansik, L., & Williams, T. J. (1983) *Biochemistry 22*, 3381–3386]. Since the overall rate of the "redox" transfer pathway of Hm^{II} from HSA to HHx is as efficient as the pathway catalyzed by the presence of 50 mM imidazole, a catalyst much more effective than this nitrogen base would have to be present in vivo to enhance the transfer of heme from HSA to HHx.

The transfer of small substrate molecules between macromolecules such as proteins and nucleic acids is a step of considerable importance in many biological processes. An example is the conservation of the iron of circulating hemin during hemolytic events through a pathway that involves the passage of the metalloporphyrin moiety from human albumin (HSA)[1] to hemopexin (HHx) (Muller-Eberhard, 1978).

Hemopexin, which is present in the serum at 1.5–2% of the concentration of albumin, binds hemin (Hm^{III}; ferriproto-

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[1] Abbreviations: HSA, human serum albumin; HHx, human hemopexin; Hm^{III}, ferriprotoporphyrin IX; Hm^{II}, ferroprotoporphyrin IX; Me_2SO, dimethyl sulfoxide; ESR, electron spin resonance; NHE, normal hydrogen electrode; emf, electromotive force.

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porphyrin IX) about 10^5 times more tightly than does the latter protein (Beaven et al., 1974; Hrkal et al., 1974). HHx functions in the uptake of Hm^{III} by hepatocytes at a specific receptor site (Smith & Morgan, 1982). In contrast, the Hm^{III}·HSA complex probably circulates until apoHHx becomes available for Hm^{III} delivery to the liver. The metalloporphyrin is then transferred to HHx, and the process of transport and degradation is continued. These two proteins bind Hm units in distinctive ways, an understanding of which is necessary for evaluation of the transfer kinetics.

The absorption spectrum of the Hm^{III}·HHx complex, unlike that of the Hm^{III}·HSA complex, displays the characteristics of a low-spin hemoprotein; e.g., there is no absorption band near 620 nm (Hrkal & Muller-Eberhard, 1971; Bearden et al., 1974, Morgan, 1976). The low-spin nature of the Hm^{III}·HHx complex has been confirmed by Mössbauer, ESR, and magnetic circular dichroism spectroscopy (Bearden et al., 1974; Morgan & Vickery, 1978). Solvent perturbation studies employing ethylene glycol indicate that the Hm^{III} chromophore is about 70% exposed to solvent when bound to hemopexin (Morgan et al., 1976a). The binding model that emerges from the combined data is one in which a single site exists for Hm^{III} having a stability constant of approximately 10^{13}–10^{14} (Hrkal et al., 1974). Regardless of the state of aggregation of Hm^{III} in solution, the final Hm^{III}·HHx complex involves a bound monomer near or at the surface of the protein molecule with two imidazole rings from histidyl moieties directly attached to the iron site. Although the reaction of monomeric Hm^{III} with HHx involves serveral steps including axial ligation of imidazole side chains of histidyl moieties, all processes subsequent to the bimolecular step [$k = 4 \times 10^7$ M^{-1} s^{-1} (Pasternack et al., 1983)] are sufficiently rapid to allow the characterization of the kinetics by a single second-order process. A plot of log k vs. $\mu^{1/2}$ is linear with a slope of −1.12, suggesting that the residual positive charge at the binding site of HHx is about 1+ to 2+. Kinetic results point to the presence of an ionizable acid group with a pK_a of 7.25 contributing to the positive charge in this region of the molecule. When this moiety is protonated, the rate constant for binding is some 3.7 times larger than when it is deprotonated.

This relatively uncomplicated description of Hm^{III} interactions with HHx can be contrasted with that determined for HSA. Although spectroscopic evidence suggests that monomeric porphyrin ligands only are bound to HSA (Moehring et al., 1983), there appears to be one high-affinity site for Hm^{III} and as many as 10 additional weaker binding sites per HSA molecule (Parr & Pasternack, 1977). The binding of porphyrins to HSA yields absorbance and fluorescence changes similar to those obtained when the uncomplexed chromophore is transferred from aqueous to organic media (Moehring et al, 1983). The combined thermodynamic and spectral results lead to the conclusion that—in contrast to HHx—the *primary* binding site for the porphyrin on HSA is in a hydrophobic region with limited access to the aqueous environment (Cannistraro, 1983). The kinetics of the reaction of Hm^{III} with HSA is more complicated than that with HHx. A second-order rate constant was determined as $k \sim 2 \times 10^5$ M^{-1} s^{-1} (Adams & Berman, 1980) with a well-defined pH dependence leading to a p$K_a \sim 5.9$ for a group near the binding site. The product of this bimolecular step then undergoes a slow, monophasic first-order process to yield the final product.

We are currently examining various chemical stratagems by which the rate of the ordinarily rather slow Hm^{III} transfer from HSA to HHx can be enhanced (Morgan et al., 1976b). One such pathway involves the mediation by a potential ligand such as imidazole for the iron site (Pasternack et al., 1983). Whereas at pH 7.0 (10 mM phosphate buffer), $\mu = 0.3$ M (NaCl), 24 °C, and $[HSA]_0/[HHx]_0 > 20$, the rate constant for the transfer of Hm^{III} is $k = 5 \times 10^{-3}$ s^{-1}, the transfer rate constant in the presence of imidazole has the form $k = (5 \times 10^{-3}) + 21[Im]^2$. In the present work we consider a second transfer pathway in which the Hm^{III} bound to HSA is first converted to heme (Hm^{II}; ferroprotoporphyrin IX). This reduction leads to an enhancement of the rate of transfer by 1–2 orders of magnitude.

Materials and Methods

HHx was purified from sera of blood donors by the method of Vretblad & Hjorth (1977). Fraction V of HSA and recrystallized hemin chloride were obtained from Miles Laboratories and Nutritional Biochemicals Corp., respectively. Dimethyl sulfoxide from Aldrich was used to determine the concentration of Hm^{III} (Collier et al., 1979). Results obtained with undistilled Me_2SO were consistent with those obtained with Me_2SO that had been vacuum distilled over BaO and stored frozen under nitrogen. All other chemicals were reagent grade and were used without further purification.

Kinetic and spectral measurements were carried out at pH 7 and $\mu = 0.3$ M unless otherwise specified. The buffer system used was 10 mM phosphate with added sodium chloride. Solutions of sodium dithionite ($Na_2S_2O_4$) were prepared by adding the solid to a buffer/sodium chloride solution previously deaerated by scrubbing with argon. The concentration of the dithionite stock was determined spectrophotometrically by the reduction of $Fe(CN)_6^{3-}$ ($\epsilon = 1026$ M^{-1} cm^{-1} at 420 nm) to $Fe(CN)_6^{4-}$ ($\epsilon = 2$ M^{-1} cm^{-1} at 420 nm). The HSA stock solution was prepared by dissolving solid HSA in previously deaerated buffer/NaCl stock. The concentration was determined spectrophotometrically at 279 nm by using $\epsilon = 3.74 \times 10^4$ M^{-1} cm^{-1} (Clark et al., 1962). HHx was stored as a concentrated, frozen solution; solutions for study were rapidly thawed. The concentration was determined from $\epsilon = 1.25 \times 10^5$ M^{-1} cm^{-1} at 280 nm (see below).

For studies of the chemical reduction of Hm^{III}·HSA or Hm^{III}·HHx small aliquots of a concentrated Hm^{III} solution in Me_2SO were added to the protein. In all cases the volume of Hm^{III} solution added was less than 2.5% of the total volume. For transfer studies, Hm^{III} was added to the HSA stock only, and sodium dithionite was added to both Hm^{III}·HSA and HHx solutions. When all the Hm^{III} had been reduced (<10 min), the kinetics of the transfer reaction were investigated. Both protein solutions were kept under a slow stream of argon across the solution surface so as to restrict oxygen and at the same time not to denature the proteins. The proteins were exposed to dithionite for a minimum amount of time for the transfer experiments. Long incubation in excess dithionite leads to porphyrin and protein degradation as evidenced by irreversible spectral changes (Morgan et al., 1976b).

Measurements were conducted at 25 °C on a Cary 14 spectrophotometer and a Durrum Model D110 stopped-flow apparatus. The kinetic data were collected digitally and analyzed by using a Cromemco Z80 microcomputer. Multiphasic data were transferred to a PRIME 750 computer which analyzed the data by using the DISCRETE program (Provencher, 1976a,b).

Electrochemical measurements were made with an optically transpartent, thin-layer electrode as described previously (Reid et al., 1982). Reduction potentials were measured against a saturated calomel reference electrode and converted to the hydrogen scale (Dutton, 1978). Data were obtained for both rat and human Hx (100 μM) with benzylviologen as mediator

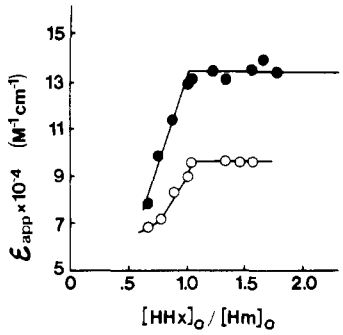

FIGURE 1: Spectrophotometric titrations of HHx with Hm^{II} (●) at 428 nm and Hm^{III} (○) at 414 nm. For each titration curve the apparent extinction coefficient, $\epsilon_{app} = Abs/[Hm]_0$, is plotted vs. $[HHx]_0/[Hm]_0$ where subscript zero signifies total concentration.

(10 μM) [pH 7.0 (phosphate), μ = 0.1 M, 20% glycerol, 25 °C] by monitoring the Soret region. Equilibration at each potential required 30–40 min. Similar measurements were attempted for the Hm^{II}·HSA complex ([heme] = 65 μM; [albumin] = 1.3 mM) with hexammineruthenium(III) chloride (Reid et al., 1982) as mediator [pH 7.0 (phosphate), μ = 0.5 M, 25 °C]. Equilibration at each potential required about 45 min.

RESULTS

Spectral titration measurements were conducted for the Hm^{II}·HHx and Hm^{III}·HHx systems. Whereas a value of $\epsilon_{280} = 1.10 \times 10^5$ M^{-1} cm^{-1} had been determined for rabbit hemopexin (Seery et al., 1972), an independent measurement of this quantity had not been made for HHx. Shown in Figure 1 are titration results for Hm^{III} and Hm^{II} with HHx assuming a value of $\epsilon_{280} = 1.25 \times 10^5$ M^{-1} cm^{-1} for the protein. Both titrations are consistent with the 1:1 stoichiometry established for rabbit and human hemopexin with porphyrins (Seery & Muller-Eberhard, 1973; Morgan et al., 1980; Gibbs et al., 1980). The titrations lead to $\epsilon_{414} = 9.7 \times 10^4$ M^{-1} cm^{-1} for Hm^{III}·HHx and $\epsilon_{428} = 1.35 \times 10^5$ M^{-1} cm^{-1} for Hm^{II}·HHx. These findings are consistent with earlier results in which reduction of Hm^{III}·HHx to Hm^{II}·HHx was shown to lead to a bathychromic shift and hyperchromism of the Soret band (Morgan et al., 1976).

Titrations at pH 7, μ = 0.3 M, involving a fixed Hm^{III} concentration of 2 μM with added HSA show changing apparent molar absorptivity until the ratio $[HSA]_0/[Hm^{III}]_0$ exceeds 20. Considering the HSA primary site only, if Hm^{III} were monomeric in solution, 99% of the porphyrin would be bound when $[HSA]_0/[Hm^{III}]_0 \sim 1.4$. Because of the extensive aggregation of Hm^{III} (Brown et al., 1981), large excesses of HSA are required for complete binding. In contrast, because of the very large stability constant for the Hm^{III}·HHx system, in spite of Hm^{III} aggregation the porphyrin is 99% bound when $[HHx]_0/[Hm^{III}]_0 \sim 1.0$.

Kinetic measurements were conducted for the dithionite reduction of Hm^{III}-HSA and of Hm^{III}·HHx. The results of these experiments are shown in Figures 2 and 3. For both systems, the concentration of dithionite was always in large excess of the Hm^{III}–protein complex. The kinetic profiles were first order over several half-lives with the observed rate constant, k_{obsd}, showing a linear dependence on $[S_2O_4^{2-}]^{1/2}$. For HSA, two series of experiments were conducted, one in which $[HSA]_0/[Hm^{III}]_0 \sim 3$ and the other in which this ratio is ~25, at which ratio all the Hm^{III} is bound. The value of k_{obsd} shows some dependence on the ratio $[HSA]_0/[Hm^{III}]_0$: $k_{obsd} = (5 \times 10^{-3}) + 0.75[S_2O_4^{2-}]^{1/2}$ at a ratio of 3 and $k_{obsd} = (2 \times 10^{-3}) + 0.25[S_2O_4^{2-}]^{1/2}$ at a ratio of 25 (all at pH 7.0, μ = 0.3 M, and 25 °C). For the reduction of Hm^{III}·HHx at pH 6.8, μ = 0.3 M, and 25 °C, the plot of k_{obsd} vs. $[S_2O_4^{2-}]^{1/2}$ (cf. Figure 3) passes through the origin with a slope of 2.5×10^3 $M^{-1/2}$ s^{-1}. A study of the pH dependence of the dithionite reduction of Hm^{III}·HHx leads to the data shown in Figure 4. The theoretical curve was obtained from the equation

$$\frac{k_{obsd}}{[S_2O_4^{2-}]^{1/2}} = \frac{(1.7 \times 10^3) + (4.7 \times 10^{10})[H^+]}{1 + (1.8 \times 10^7)[H^+]} \quad (1)$$

The calculated value of $k_{obsd}/[S_2O_4^{2-}]^{1/2}$ at pH 6.8 from this equation is 2.4×10^3 $M^{-1/2}$ s^{-1} compared to the experimental value of 2.5×10^3 $M^{-1/2}$ s^{-1}.

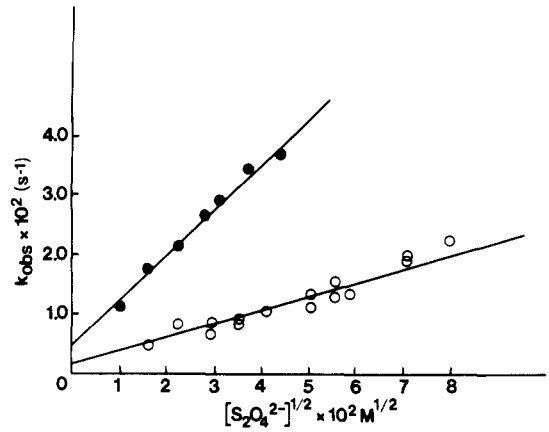

FIGURE 2: Kinetics results for the reduction of Hm^{III}·HSA by dithionite. Experiments were conducted under pseudo-first-order conditions in the presence of excess dithionite. Two sets of experiments were conducted in which the initial albumin to hemin ratios were about 3 (●) and 25 (○), respectively.

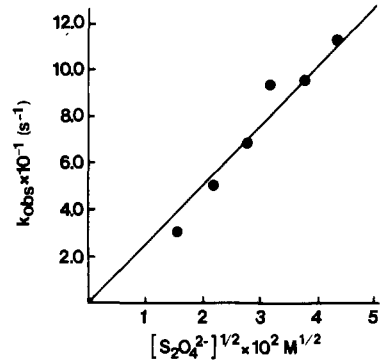

FIGURE 3: Kinetics results for the reduction of Hm^{III}·HHx by dithionite. Experiments were conducted under pseudo-first-order conditions in the presence of excess dithionite, $[Hm^{III}]_0$ = 2.5 μM, and $[HHx]_0$ = 3.2 μM.

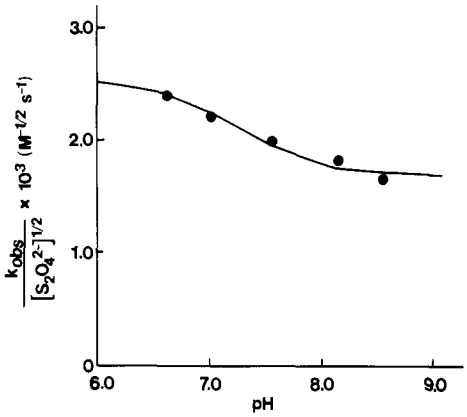

FIGURE 4: Study of the pH dependence of the reduction rate of Hm^{III}·HHx.

Table I: Transfer Kinetics of Heme (Hm^{II}) from Albumin to Hemopexin:[a]

$$Hm^{II}\cdot HSA + HHx \rightarrow Hm^{II}\cdot HHx + HSA$$

$[Hm^{II}\cdot HSA]$	[HSA]	[HHx]	pH	k_f (s^{-1})	k_s (s^{-1})
3.0×10^{-6}	6.0×10^{-5}	3.0×10^{-6}	6.98	0.24	0.016
3.0×10^{-6}	1.2×10^{-4}	3.0×10^{-6}	6.98	0.30	
1.0×10^{-6}	2.0×10^{-5}	1.0×10^{-6}	5.68	0.43	0.12
1.0×10^{-6}	2.0×10^{-5}	1.0×10^{-6}	6.02	0.47	0.12
1.0×10^{-6}	2.0×10^{-5}	1.0×10^{-6}	6.53	0.26	0.056
1.0×10^{-6}	2.0×10^{-5}	1.0×10^{-6}	6.80	0.23	0.033
1.0×10^{-6}	2.0×10^{-5}	1.0×10^{-6}	7.02	0.19	0.028

[a] Conditions: μ = 0.3 M, 25 °C, and λ 428 nm.

Table II: Reduction Potentials of the (Hm^{III}) Hemin–Hemopexin Complex

source	t (°C)	E_m° vs. NHE (mV)	Nernst slope (mV)
rat	9.0	−200 ± 3	67 ± 2
rat	20.8	−207 ± 3	66 ± 3
rat	30.0	−211 ± 3	65 ± 3
human	10.2	−183 ± 3	73 ± 4
human	20.0	−204 ± 3	76 ± 3
human	30.0	−202 ± 3	65 ± 1

The transfer of Hm^{II} from HSA to HHx was studied at μ = 0.3 M and 25 °C; a summary of the results of these experiments is shown in Table I. The kinetics are biphasic and could be analyzed as two first-order processes yielding rate constants k_f and k_s whose values are independent of $[Hm^{III}\cdot HSA]_0$, $[HSA]_0$, and $[HHx]_0$. However, there is a slight dependence on pH and ionic strength. Under the above conditions (μ = 0.3 M, 25 °C) the values obtained at pH 7 are $k_f \sim 0.2\ s^{-1}$ and $k_s \sim 0.03\ s^{-1}$. The dependence of k_f and k_s on pH can be summarized by an equation of the form

$$k_{obsd} = \frac{a[H^+] + b}{c[H^+] + 1} \qquad (2)$$

For the fast effect (k_f), $a = 8.2 \times 10^5\ M^{-1}\ s^{-1}$, $b = 0.15\ s^{-1}$, and $c = 1.6 \times 10^6\ M^{-1}$; for the slow effect ($k_s$), $a = 2.8 \times 10^5\ M^{-1}\ s^{-1}$, $b = 0.013\ s^{-1}$, and $c = 1.7 \times 10^6\ M^{-1}$. Values for k_f and k_s calculated at pH 7 are $0.20\ s^{-1}$ (observed $0.2\ s^{-1}$) and $0.03\ s^{-1}$ (observed $0.03\ s^{-1}$). The dependence of the transfer rate constants on ionic strength was explored. Plots of log k vs. $\mu^{1/2}$ yield parallel lines of slope 0.59 for the fast and slow processes.

The results from the electrochemical experiments on the Hm^{III}·HHx complex are shown in Table II. As can be seen, the data obtained for the rat protein were of consistently better quality than those obtained for the human protein. The basis for the deviation of the Nernst slopes from the theoretically expected value of 60 mV is not clear but may be a function of the particular protein preparation used. Alternatively, this deviation may arise from secondary heme binding sites, the nature of which may differ between the two species. From the present results, it appears that the reduction potential for the principal Hm^{III}·HHx complex is the same for the rat and human proteins, and we estimate a value for this potential of −209 mV at 25 °C and pH 7 from the data for the rat Hx. The spectroelectrochemical measurements for the Hm^{III}·HSA complex were complicated by a shift in the observed isosbestic points during the titration which prevented satisfactory numerical analysis. Qualitatively, however, it appeared that the midpoint potential for this couple was approximately −100 mV vs. NHE. Presumably, the failure to observe isosbesty arose from the presence of more than one type of Hm^{III}·HSA complex at varying reduction potentials.

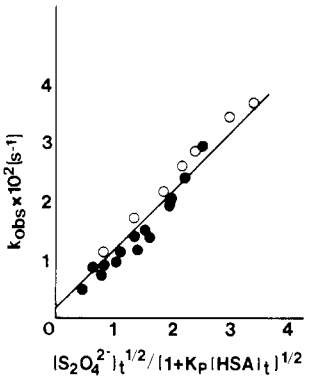

FIGURE 5: Plot of the kinetics results obtained for the reduction of Hm^{III}·HSA by dithionite in which the binding of $S_2O_4^{2-}$ to HSA is considered. The two data sets of Figure 2 now lie on the same line. As earlier, the filled circles represent data in which the initial albumin to heme ratio is 3, and the open circles are for data at a ratio of 25.

Discussion

The kinetic results for the reduction of the two Hm^{III}–protein complexes are consistent with a mechanism involving the dissociation of dithionite into SO_2^- radicals. However, several important features of the reduction should be considered before a direct comparison can be made of the results obtained for HSA and HHx.

As indicated earlier, the aggregation of Hm^{III} and the size of the Hm^{III}·HSA stability constant require excess HSA for the binding of appreciable fractions of Hm^{III}. The kinetic results (cf. Figure 2) for the reduction of Hm^{III}·HSA show a dependence of k_{obsd} on $[HSA]_0/[Hm^{III}]_0$. It may be recalled that albumin has a large number of nonspecific binding sites for fatty acid residues, porphyrins, and other anions (Parr & Pasternack, 1977). The kinetic analysis for the reduction should take into account that some of the dithionite dianion can bind to these nonspecific sites:

$$S_2O_4^{2-} + HSA_t \rightleftharpoons S_2O_4^{2-}\cdot HSA \qquad K_p$$

$$S_2O_4^{2-} \rightleftharpoons 2SO_2^- \qquad K_d$$

$$SO_2^- + Hm^{III}\cdot HSA \xrightarrow{k_1} Hm^{II}\cdot HSA + \text{sulfur products}$$

where the subscript "t" represents the total concentration in all forms.

$$\text{rate} = k_1[SO_2^-][Hm^{III}\cdot HSA]$$

This leads to

$$\text{rate} = k_1\left(\frac{K_d}{1 + K_p[HSA]_t}\right)^{1/2}[S_2O_4^{2-}]_t^{1/2}[Hm^{III}\cdot HSA] \qquad (3)$$

where $[S_2O_4^{2-}]_t = [S_2O_4^{2-}] + [S_2O_4^{2-}\cdot HSA]$. Then

$$k_{obsd} = \frac{k_1K_d^{1/2}[S_2O_4^{2-}]_t^{1/2}}{(1 + K_p[HSA]_t)^{1/2}} \qquad (4)$$

A plot of k_{obsd} vs. $[S_2O_4^{2-}]_t^{1/2}/(1 + K_p[HSA]_t)^{1/2}$ is linear (cf. Figure 5) for $K_p = 2.4 \times 10^4\ M^{-1}$ with all the kinetic data lying on a line having the equation

$$k_{obsd}^{corr} = k_{obsd}(1 + K_p[HSA]_t)^{1/2} = (2 \times 10^{-3}) + 1.0[S_2O_4^{2-}]_t^{1/2} \qquad (5)$$

The slope of this line, $k_1K_d^{1/2} = 1.0\ M^{-1/2}\ s^{-1}$. However, the nonzero intercept requires a further modification of the mechanism. The stability constant for Hm^{III}·HSA is suffi-

ciently small that one must consider dissociation of the complex with subsequent *rapid* reduction by dithionite. Dithionite reduces uncomplexed Hm^{III} much more rapidly than it does Hm^{III}·HSA (Cassatt et al., 1977). Thus, the mechanism should include both reduction pathways:

$$Hm^{III}\cdot HSA + SO_2^- \xrightarrow{k_1} Hm^{II}\cdot HSA + \text{sulfur products}$$

$$Hm^{III}\cdot HSA \xrightarrow{k_1'} Hm^{III} + HSA$$

$$Hm^{III} + SO_2^- \xrightarrow{\text{very fast}} Hm^{II} + \text{sulfur products}$$

$$Hm^{II} + HSA \xrightarrow{\text{very fast}} Hm^{II}\cdot HSA$$

Then

$$k_{obsd}^{corr} = k_1' + k_1 K_d^{1/2}[S_2O_4^{2-}]_t^{1/2}$$

This leads to $k_1' = 2 \times 10^{-3}$ s^{-1}, and for $K_d = 1.4 \times 10^{-9}$ M (Mehrotra & Wilkins, 1980), $k_1 = 2.7 \times 10^4$ M^{-1} s^{-1}. The rate constant k_1' for the dissociation of Hm^{III}·HSA determined here is in good agreement with the value determined earlier under similar experimental conditions (Pasternack et al., 1983).

The reduction of Hm^{III}·HHx by dithionite is consistent with the mechanism

$$S_2O_4^{2-} \rightleftharpoons 2SO_2^- \qquad K_d$$

$$Hm^{III}\cdot HHx + SO_2^- \xrightarrow{k_2} Hm^{II}\cdot HHx + \text{sulfur products}$$

which leads to

$$k_{obsd} = k_2 K_d^{1/2}[S_2O_4^{2-}]_t^{1/2}$$

Because of the very large stability constant for Hm^{III}·HHx, there is virtually no dissociation of this complex, and we obtain an intercept indistinguishably different from zero. The value of k_2 derived from the mechanism and these data is 6.7×10^7 M^{-1} s^{-1}.

The relative Marcus theory can be applied to these redox reactions and the results interpreted by using estimates for the self-exchange rate constants for low-spin Hm^{III}·HHx and high-spin Hm^{III}·HSA (Pasternack & Spiro, 1978). The driving force is known for the HHx case and can be estimated for the HSA system; the reduction potentials are (Mehrotra & Wilkins, 1980)

$$-209 \text{ mV for } e^- + Hm^{III}\cdot HHx \rightharpoonup Hm^{II}\cdot HHx$$

$$\sim -100 \text{ mV for } e^- + Hm^{III}\cdot HSA \rightharpoonup Hm^{II}\cdot HSA$$

$$-660 \text{ mV for } e^- + HSO_3^- \rightharpoonup SO_2^- + OH^- \text{ at pH 7}$$

The value given here for the reduction of Hm^{III}·HHx is considerably at variance with a published value of +64 mV (Hrkal et al., 1977). There is no obvious reason for this discrepancy; it may arise either from differences in the measurement techniques used or from differences between the protein samples analyzed. However, if the material studied here did have an actual reduction potential in the range 0–100 mV, then the rate of equilibration of the protein with the working electrode during the potentiometric titrations should have been much faster, as we have observed previously with native and heme-substituted derivatives of cytochrome b_5 (Reid et al., 1982, 1984; Reid, 1984). A low value for the reduction potential of Hm^{III}·HHx may reflect a relatively hydrophilic heme environment (Kassner, 1972) or a relatively high degree of heme exposure (Stellwagen, 1978). We note that these two possibilities are not necessarily mutually exclusive.

We apply the Marcus theory to the kinetic results by considering the ratio of the rate constants for reduction of Hm^{III}·HHx and Hm^{III}·HSA. The Marcus equation simplifies thereby in that neither the self-exchange rate constant for dithionite nor the emf for the dithionite half-reaction appears in the expression other than in the relatively insensitive factor, f:

$$\frac{k_{12}^{HHx}}{k_{12}^{HSA}} = \left(\frac{k_{22}^{HHx}}{k_{22}^{HSA}}\frac{K_{12}^{HHx}}{K_{12}^{HSA}}\frac{f^{HHx}}{f^{HSA}}\right)^{1/2}$$

with

$$\log (K_{12}^{HHx}/K_{12}^{HSA}) = 16.9(\epsilon_{HHx}^0 - \epsilon_{HSA}^0)$$

and

$$\log f = (\log K_{12})^2/4 \log (k_{11}k_{22}/Z^2)$$

Using estimates of $k_{11} \sim 10^{-3}$ M^{-1} s^{-1} for the self-exchange rate constant of dithionite (Mehrotra & Wilkins, 1980) and 0.014 for $K_{12}^{HHx}/K_{12}^{HSA}$, we obtain $k_{22}^{HHx}/k_{22}^{HSA} \sim 10^8$. From previous work (Pasternack & Spiro, 1978) we anticipate that the self-exchange rate constants for iron porphyrins that are low spin and in-plane will be larger by 3–4 orders of magnitude than for ones that are high spin and out-of-plane. The additional 4–5 orders of magnitude observed in this system may well reflect the differences in accessibility of the two Hm^{III} sites to the reducing agent. Whereas Hm^{III} is to be found at the surface of hemopexin (Morgan et al., 1976a), it is located in a more interior hydrophobic region of albumin (Cannistraro, 1983).

The pH dependence of the rate constant for the reduction of Hm^{III}·HHx by dithionite is interpreted as being due to an ionizable group on the protein in the vicinity of the binding site. Such a moiety, having a pK_a of 7.25, was found to play a role in the binding kinetics of Hm^{III} with hemopexin (Pasternack et al., 1983). Standard kinetic analysis leads to

$$\frac{k_{obsd}}{[S_2O_4^{2-}]^{1/2}} = \frac{k_{02}' + k_{02}[H^+]/K_a}{1 + [H^+]/K_a} \qquad (6)$$

where k_{02} and k_{02}' are the rate constants for the reduction of the protonated and deprotonated forms of the Hm^{III}·HHx complex, respectively, and K_a is the acid dissociation constant of the ionizable group. From the results shown in eq 1, $k_{02} = 2.6 \times 10^3$ M$^{-1/2}$ s^{-1}, $k_{02}' = 1.7 \times 10^3$ M$^{-1/2}$ s^{-1}, and pK_a = 7.25, in excellent agreement with the pK_a obtained earlier (Pasternack et al., 1983).

The transfer of Hm^{II} from HSA to HHx gives rise to biphasic kinetics involving two first-order processes having rate constants of $k_f = 0.3$ s^{-1} and $k_s = 0.02$ s^{-1} at pH 7, $\mu = 0.3$ M, and 25 °C. It is not unequivocally clear whether this transfer involves two sequential steps of comparable rate

$$Hm^{II}\cdot HSA + HHx \rightharpoonup I \rightharpoonup Hm^{II}\cdot HHx + HSA \qquad (7)$$

or two parallel pathways

$$Hm^{II}\cdot HSA + HHx \rightleftharpoons Hm^{II}\cdot HHx + HSA \qquad (8)$$

The pH and ionic strength dependencies of the rate constants favor the parallel pathway interpretation. Both phases of the transfer have pH dependencies that can be fit by eq 2. Using a model similar to the one discussed above for eq 6, we obtain pK_a = 6.0 and 6.2 for the fast and slow steps, respectively. That the critical pK_a is similar for the fast and slow steps (and is about the same as for the binding kinetics of Hm^{III} to HSA) suggests a similar rate-determining process for both—the dissociation of the Hm^{II}·HSA complex. Furthermore, the dependence of $-\log k$ vs. $\mu^{1/2}$ yields two parallel lines having

a positive slope, again suggesting a similar kinetic process for both effects as, for example, the separation of the negatively charged heme group from the positively charged binding region of the protein.

Can an explanation be offered as to why the transfer might involve parallel pathways? Quite recent results provide evidence that HSA exists in solution as two stable, non- (or very slowly) interconverting conformers whose relative concentrations are unaffected by pH, source of the protein, presence of fatty acids, or state of aggregation (Moehring et al., 1983). Conformer I is twice as plentiful as conformer II with binding constants for deuteroporphyrin IX determined as 1.5×10^7 M^{-1} and 4.9×10^8 M^{-1}, respectively. The secondary sites on both conformers have stability constants $\leq 5 \times 10^5$ M^{-1}. Thus, for a population of deuteroporphyrin IX, the apparent stability constant for the primary site on HSA would be $\bar{K} \sim 2 \times 10^8$ M^{-1}. The value of $\bar{K}$ determined for Hm^{III} is about 5×10^7 M^{-1} (Hrkal et al., 1981). There have been no obvious manifestations of the existence of the two HSA conformers in reaction kinetic studies thus far reported in the literature. However, it is possible that Hm^{II} dissociates from HSA at slightly different rates depending on the conformer to which it is bound.

Finally, to compare the relative efficiency of a "ligand-mediated" pathway to a "redox" pathway for transfer of the metalloporphyrin from HSA to HHx, we calculate that for [imidazole] $\geq$ 50 mM the rates for the two processes become comparable. Thus, for the ligand-mediated transfer to compete with the redox pathway in vivo, a catalyst much more efficient than imidazole would have to be present.

Registry No. Hemin, 16009-13-5; heme, 14875-96-8.

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