The Association Rate Constant for Heme Binding to Globin Is Independent of Protein Structure†

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ABSTRACT: Rate constants for CO-heme binding to 35 different recombinant apomyoglobins and several other apoproteins were measured in an effort to understand the factors governing heme affinity and the velocity of the association reaction. Surprisingly, the rate constant for the binding of monomeric heme is $\approx 1 \times 10^8$ M^{-1} s^{-1} regardless of the structure or overall affinity of the apoprotein for iron−porphyrin. Major differences between the proteins are reflected primarily in the rates of dissociation of the prosthetic group. Slow phases observed in the reaction of CO heme with excess apomyoglobin result from formation of nonspecific heme−protein complexes which must dissociate before heme can bind specifically in the heme pocket. Once the specific heme−globin complex is formed, the heme pocket rapidly collapses around the porphyrin, simultaneously forming the bond between the proximal His^{93} and the heme iron atom. The overall affinity of sperm whale apomyoglobin for hemin is $\sim 1 \times 10^{14}$ M^{-1}. Nonspecific hydrophobic interactions between the porphyrin and the apolar heme cavity account for a factor of 10^5−10^7. Covalent bond formation between Fe^{3+} and His^{93}(F8) provides an additional factor of 10^3−10^4. Specific interactions with conserved amino acids in the heme pocket contribute the final factor of 10^3−10^4.

The affinities of apohemoglobins and apomyoglobins for heme[1] are very large, showing equilibrium dissociation constants in the 10^{-12}−10^{-15} M region. In the structures of the holoproteins, the prosthetic group appears to be stabilized by a large number of hydrophobic, apolar, and electrostatic contacts. The vinyl groups are pointing toward the protein interior and surrounded by apolar aliphatic and aromatic side chains, whereas the propionates point toward the solvent and interact with a variety of charged or polar amino acids. These interactions and the extremely small dissociation constants imply a high degree of specificity in the binding process. However, a variety of experimental evidence suggests that the association of heme with apoglobin is little affected by globin structure. (1) Gibson and Antonini (1963) showed that at pH 9.1 the CO forms of meso-, deutero-, and hematohemes react with apohemoglobin at rates which are only 2−4 times smaller than that for CO protoheme binding, and Rose and Olson (1983) reported similar results at pH 7.2. (2) Chu and Bucci (1979) showed that dimethyl ester CO-heme reacts with apohemoglobin at the same rate as CO protoheme. (3) La Mar *et al*. (1983, 1984) have shown that rapid insertion of protoheme into apomyoglobin results in a 50/50 mixture of two distinct orientations that differ by 180° rotation about the $\alpha-\gamma$ axis of the porphyrin ring. Initial holomyoglobin formation is then followed by a slow reorientation process which results in an equilibrium distribution of greater than 95% of the conformer that is seen by X-ray crystallography. All of these results imply that specific heme−globin interactions do not play a significant role in regulating the rapid, bimolecular association of heme with apoglobins.

In order to examine more directly the role of globin structure, we have measured association rate constants for CO-heme binding to a large number of apomyoglobin mutants with substitutions in the heme pocket. Many of the replacements were chosen because they had previously been shown to affect the rate of hemin dissociation and the stability of the corresponding apoprotein (Hargrove *et al*., 1994a,b). The contributions of nonspecific hydrophobic interactions and the His^{93}−iron bond to the overall affinity of myoglobin for heme were estimated by comparing association and dissociation rate constants for heme binding to wild-type sperm whale myoglobin with those for binding to $His^{93} \rightarrow$ Gly apomyoglobin and bovine serum albumin.

The bimolecular reaction of heme with globin often shows multiple phases which vary greatly in rate and amplitude depending on the oxidation and ligand state of the iron atom. Gibson and Antonini (1960) provided the first comprehensive kinetic models for these reactions. The simplest kinetic behavior was observed for the binding of monomeric CO-heme to apohemoglobin. This reaction shows large, rapid absorbance changes with an apparent association rate constant equal to $\sim 5 \times 10^7$ M^{-1} s^{-1}. The following model was proposed to account for the dependence of the observed rate constant on apoprotein concentration:

$$\text{CO-heme} + \text{globin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{complex} \xrightarrow{k_2} \text{holoprotein} \quad (1)$$

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[1] The names of the ferric and ferrous forms of iron−porphyrin complexes are as follows: heme (Fe^{2+}, no ligand bound), CO-heme (Fe^{2+}, 5-coordinate with carbon monoxide), hemin (Fe^{3+}, 5-coordinate with weakly bound water), and dicyanohemin (Fe^{3+}, 6-coordinate with two cyanides).

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The initial bimolecular process involves very rapid formation of a reversible, nonspecific complex with k_1 and $k_{-1} \geq \sim 1 \times 10^8$ M^{-1} s^{-1} and ~ 1000 s^{-1}, respectively, for human apohemoglobin. First-order formation of the final CO-hemoglobin complex appears to occur at a rate of ~ 500 s^{-1} at pH 7.0, 20 °C. Rose and Olson (1983) obtained similar results at pH 7.2 . They argued that the first step is sufficiently rapid to be considered in equilibrium throughout the binding process and that, in the presence of excess apoprotein, the observed rate constant is given by k_2[Gb]/(K_d + [Gb]), where [Gb] is the concentration of apoglobin and $K_d = k_{-1}/k_1$. Under most experimental conditions, there is a linear dependence of the observed rate on [Gb], and the apparent association rate constant, k_H' (slope of k_{obs} vs [Gb]), is given by k_2k_1/k_{-1}.

In contrast to CO-heme, the reaction of aquohemin with globin shows multiple phases that have been attributed to multiple aggregation states of the prosthetic group in aqueous solvents (Gibson & Antonini, 1960; Adams, 1977; Kawamura-Konishi & Suzuki, 1985). The reaction of dicyanohemin with globin is first-order, monophasic, and very slow, ~ 0.01 s^{-1} at room temperature. The unusually slow rate was originally attributed to the interconversion between di- and monocyanohemin prior to reaction with globin (Gibson & Antonini, 1960; Brown *et al*., 1970). However, Leutzinger and Beychok (1981) and Kawamura-Konishi *et al*. (1988) have shown that dicyanohemin rapidly quenches the fluorescence of apohemoglobin α subunits and apomyoglobin with bimolecular rate constants equal to $\sim 5 \times 10^7$ M^{-1} s^{-1}. The slow dicyanohemin absorbance changes were interpreted as due to either slow folding events or displacement of a cyanide ligand by the proximal histidine.

We have reexamined the rates of hemin, CO-heme, and dicyanohemin binding to apomyoglobin using both absorbance and fluorescence techniques. Our goals were to test the kinetic mechanisms proposed by Gibson and Antonini (1960) and Kawamura-Konishi *et al*. (1988) and to establish the conditions for screening the kinetics of heme binding to apomyoglobin mutants.

MATERIALS AND METHODS

Production of Proteins. Sperm whale, horse, and sheep native myoglobins were purchased from Sigma. Wild-type sperm whale myoglobin and mutants at positions 29, 43, 64, 68, and 107 were made as described in Hargrove *et al.* (1994b). Mutants at positions 32, 45, 89, and 97 were made using oligonucleotide-directed mutagenesis starting with the synthetic sperm whale myoglobin gene developed by Springer and Sligar (1987). Recombinant V68T pig myoglobin was obtained from Anthony J. Wilkinson at York University, York, U.K. (Smerdon *et al*., 1991). Native leghemoglobin *a* (Lba) was provided by Gautam Sarath at the University of Nebraska−Lincoln. H93G sperm whale myoglobin was purified as described by Barrick (1994). This mutant is prepared in the presence of external imidazole. The aquomet form contains hemin coordinated with imidazole which is noncovalently bound in the proximal portion of the heme pocket (Barrick, 1994). Human α and β chains were prepared by Antony J. Mathews at Somatogen, Inc., Boulder, CO, using the method of Bucci (1981). Human H64V/V68H was expressed and purified at Case Western Reserve University following the method of Ikeda-Saito *et al*. (1991). Apomyoglobins were prepared using the methyl ethyl ketone extraction method described by Ascoli *et al*. (1981) and Hargrove *et al*. (1994a). Bovine serum albumin was purchased from Sigma and used without further purification.

Preparation of Reaction Solutions. The buffer used in all of the heme association reactions was 50 mM Tris and 50 mM NaCl, pH 8.0 (Light & Olson, 1990). Under these conditions heme aggregation and nonspecific binding are at a minimum. Reactions at lower pH showed larger slow phases associated with aggregated states of the heme. All heme containing solutions were prepared and used within 3 h to minimize oxidation and precipitation.

Stock hemin (Sigma) solutions were prepared by dissolving a small amount ($\sim$5 mg) in $\sim$1 mL of 0.1 M NaOH. This solution was then passed through a 0.2-μm filter into an Eppendorf tube. For the production of CO-heme, the buffer was preequilibrated with 100% CO gas for 20 min. One milliliter of this buffer was drawn into a 5-mL Hamilton gastight syringe and several granules of dry sodium dithionite were added. Approximately 200 μL of the filtered hemin solution was added to the solution, and the concentration of CO-heme was determined spectrophotometrically using ϵ_{407} = 147 mM^{-1} cm^{-1} (Light, 1987). This solution (usually 100−200 μM) was used as a stock for dilution to the appropriate reactant concentration.

Hemin solutions of known concentration were prepared by adding approximately 200 μL of the filtered stock solution to 1 mL of air-equilibrated buffer. A small aliquot of this solution was added to a cuvette containing CO-equilibrated buffer and dithionite, and the observed concentration of CO-heme was used to compute the concentration of the original stock hemin solution. Stock deoxyheme solutions were prepared in a similar fashion, except that all buffers were bubbled with 100% nitrogen for 20 min, and sodium dithionite was added to keep the hemin reduced. In the experiments involving dicyanohemin, all reaction solutions contained 10 mM KCN to ensure that free hemin was in the dicyano form. Higher concentrations of KCN had no effect on the absorbance changes or reaction kinetics.

The concentrations of most apomyoglobin solutions, apoleghemoglobin, and human β apoglobin were determined spectrophotometrically using ϵ_{280} = 15.2 mM^{-1} cm^{-1} (Light, 1987). ϵ_{280} = 22.8 mM^{-1} cm^{-1} was used for V68W and L89W apomyoglobins to account for the additional tryptophan, and ϵ_{280} = 7.6 mM^{-1} cm^{-1} was used for human α apoglobin which contains only one tryptophan. Bovine serum albumin solutions were prepared by weight.

Stopped-Flow Measurements. Stopped-flow absorbance measurements were made with a Gibson-Dionex stopped-flow apparatus equipped with an OLIS data acquisition system. The reactions with CO-heme, hemin, dicyanohemin, and deoxyheme were monitored at 423, 409, 421, and 432 nm, respectively. Fluorescence measurements were made with a SLM 8100 spectrofluorometer equipped with a Milliflow stopped-flow attachment. Excitation was at 285 nm, and total fluorescence emission was measured using a 320-nm cutoff filter. Reactions with CO and deoxyheme were not measured by fluorescence due to the strong UV absorbance of dithionite. All reactions, except for those in Figure 2B, were carried out with excess apoglobin. At apoglobin concentrations greater than 5 μM, a significant portion of the reaction is lost in the 3-ms dead time of the apparatus.

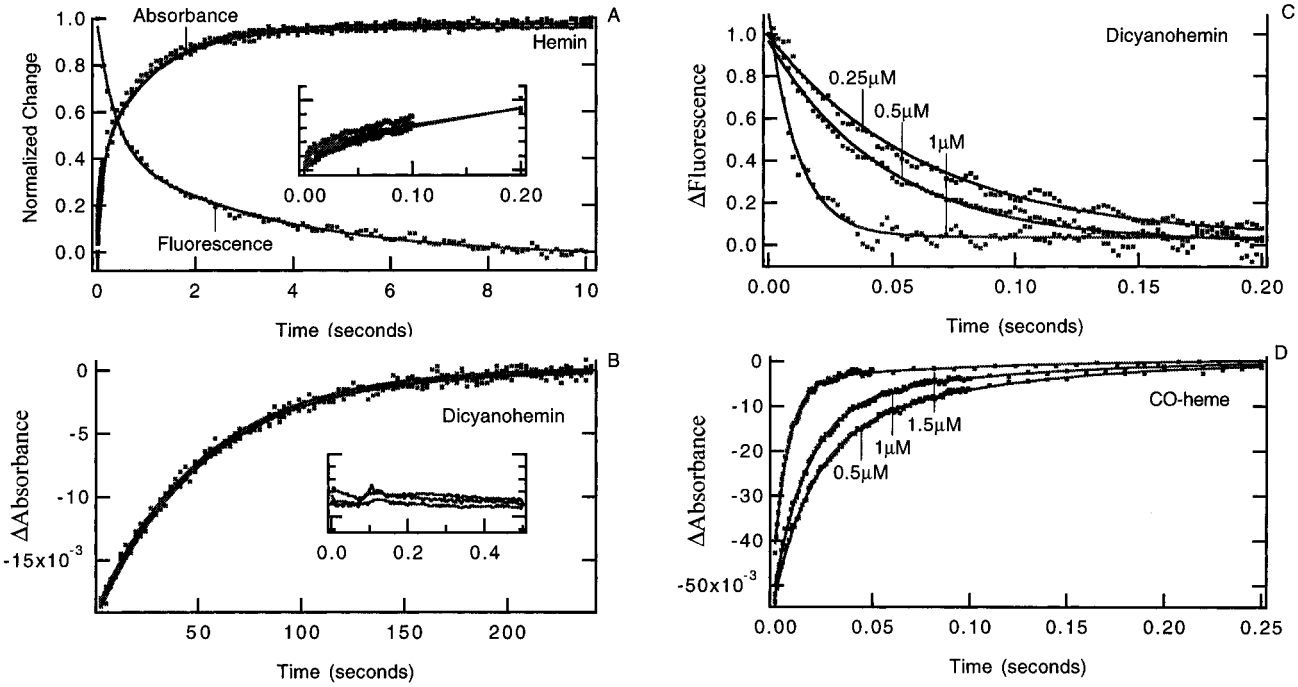


FIGURE 1: Time courses for heme binding to apomyoglobin. (A) Hemin binding to wild type apomyoglobin. The reactions of 0.25 μM hemin with 0.5, 1, and 2 μM apomyoglobin were monitored by both fluorescence and absorbance changes. Three absorbance traces (filled circles) are shown in the panel A and superimpose almost exactly (only one fitted line is shown). For clarity, only the fluorescence trace at 2 μM globin is shown. All six time courses are biphasic with pseudo-first-order rates of $\approx$ 9 and 0.9 s^{-1}. The inset is an expanded view of the early absorbance time points showing that there is a very small (5−10%) bimolecular fast ($k_H' \geq 10^7$ M^{-1} s^{-1}) phase associated with hemin binding. (B) Dicyanohemin binding to apomyoglobin as monitored by absorbance changes. Apomyoglobin at 0.5, 1, and 2.5 μM was reacted with 0.25 μM dicyanohemin. In each case the reaction was monophasic with an observed first-order rate constant equal to 0.019 s^{-1}. The three time courses superimpose almost exactly. The inset is an expanded view of the early time points showing that no fast reaction occurs. (C) Dicyanohemin binding to apomyoglobin as monitored by fluorescence changes. Time courses for the reaction of 0.25, 0.5, and 1 μM apomyoglobin with 0.25 μM dicyanohemin were fitted to a single-exponential expression. A plot of k_{obs} vs [globin] gave a bimolecular association rate constant of 5×10^7 M^{-1} s^{-1}. No fluorescence changes were observed over a 5-min period following these reactions. (D) CO-Heme binding to apomyoglobin. Time courses for the reaction of 0.5, 1, and 2.5 μM wild-type apomyoglobin with 0.25 μM CO-heme were fitted to a two-exponential expression. The rate constant for the larger phase depended linearly on [globin] and gave an apparent bimolecular association rate constant equal to 7×10^7 M^{-1} s^{-1}. The small slow phase (5−15%) appeared to be first-order with a rate constant equal to $\sim$9 s^{-1}. These panels show that monomeric heme binds rapidly to apomyoglobin by a second-order process.

Data were fitted using the data analysis program Igor-Pro (Wavematics, Inc.).

Hemin Dissociation Experiments. Hemin loss from H93G myoglobin and bovine serum albumin (BSA) was measured by stopped-flow techniques using the H64Y/V68F apomyoglobin assay described by Hargrove *et al.* (1994a). For reactions involving the ferric H93G metmyoglobin−imidazole complex, 12 μM holoprotein was mixed with 60 μM H64Y/V68F apoglobin at 37 °C, and the reaction was monitored as a decrease in absorbance at 410 nm.

Rates of hemin and CO-heme dissociation from BSA were measured in the following way: 60 μM BSA preequilibrated with 12 μM hemin or CO-heme was mixed with 60 μM apo-H64Y/V68F and the reaction was measured in the stopped-flow apparatus as an increase in absorbance at 423 nm (for CO-heme) or 600 nm (for the formation of H64Y/V68F metMb). All reaction solutions in the CO-heme dissociation experiment were equilibrated with 100% CO and contained a small amount of sodium dithionite to prevent oxidation. Hemin and CO-heme dissociation from BSA was measured at 20 °C for direct comparison with heme association rates.

RESULTS

Reaction of Heme with Apomyoglobin. Rates of hemin, dicyanohemin, and CO-heme binding to apomyoglobin were measured, and the results are similar to those reported by Gibson and Antonini (1960) for the same reactions with apohemoglobin. Figure 1A shows the changes in absorbance and fluorescence associated with hemin binding to apomyoglobin. The fluorescence of apomyoglobin is quenched nearly 50-fold upon hemin binding. The normalized absorbance and fluorescence time courses overlay fairly well and show that the majority of the reaction is very slow and has little dependence on globin concentration. Presumably, slow dissociation of hemin aggregates is required before the prosthetic group can interact either specifically or nonspecifically with apomyoglobin.

Dicyanohemin binding to apomyoglobin appears to be a slow first-order process with a rate constant $\approx$ 0.02 s^{-1} when measured by Soret absorbance changes (Figure 1B). However, the fluorescence time course for the same reaction shows a rapid bimolecular process with a rate constant of $\approx$ 5×10^7 M^{-1} s^{-1} (Figure 1C). Similar time courses were obtained by Leutzinger and Beychok (1981) for the reaction of dicyanohemin with isolated human apohemoglobin α chains and by Kawamura-Konishi *et al.* (1988) for the same reaction with horse heart apomyoglobin. All of these results show that dicyanohemin binds to apoglobin as rapidly as monomeric CO-heme, immediately quenching most of the tryptophan fluorescence. However, little or no hemin

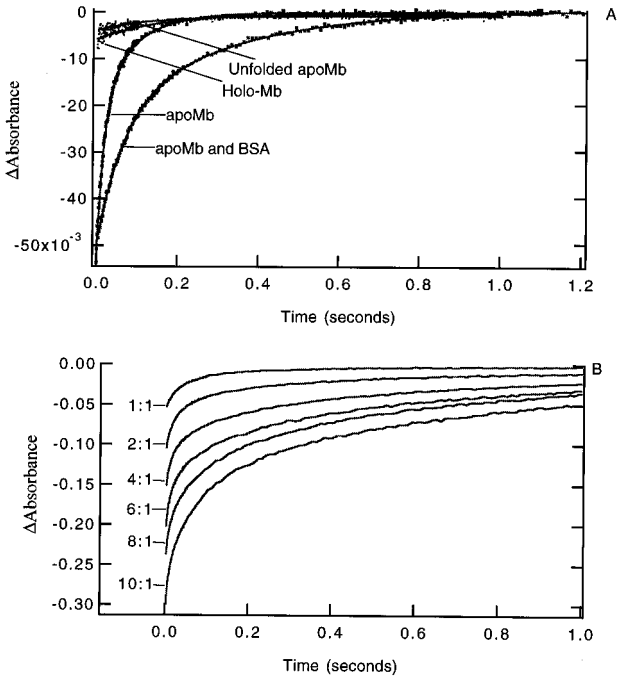


FIGURE 2: Nonspecific interactions account for the slow phase of CO-heme binding. (A) CO-Heme binding to myoglobin in the presence of excess protein. The reaction of 0.25 μM heme and 0.5 μM apomyoglobin is very rapid and almost monophasic with a pseudo-first-order rate constant of $\sim$ 35 s^{-1} (apoMb trace). The addition of 5 μM bovine serum albumin to this reaction mixture causes much slower CO-heme binding (apoMb and BSA trace). The reactions of 0.25 μM CO-heme with 0.5 μM CO-myoglobin or with 0.5 μM denatured apomyoglobin in the presence of 5 M GdmCl show only small, slow phases with first-order rate constants $\approx$ 9 s^{-1} (holo-Mb and unfolded ApoMb traces). (B) Binding reactions in the presence of excess CO-heme. In this experiment, 0.5 μM apomyoglobin was reacted with increasing concentrations of CO-heme. The ratio of heme/globin is shown next to the corresponding time course. The amplitude of the slow phase increases in proportion to the concentration of excess heme, showing that nonspecific interactions of CO-heme with apomyoglobin can result in absorbance changes similar to those observed for the formation of native holoprotein.

absorbance change is associated with this initial binding process. Kawamura-Konishi *et al*. (1988) argued convincingly that the large slow absorbance change is due to displacement of the proximal cyanide ligand by the imidazole side chain of His^{93}(F8), although a slow first-order migration of cyanohemin from the protein surface to the interior of the heme pocket cannot be ruled out.

Figure 1D shows absorbance time courses for the reaction of CO-heme with wild-type apomyoglobin. Each trace fits to two phases: one large bimolecular phase with a second-order rate constant = 7 $\times$ 10^7 M^{-1} s^{-1} and one small first-order phase with a rate $\approx$ 9 s^{-1}. The fast phase represents the combination of heme with apomyoglobin to form holo CO-myoglobin. Figure 2 suggests that the slow process represents nonspecific binding of heme to globin. In Figure 2A, 0.25 μM CO-heme was reacted with 0.5 μM apomyoglobin resulting in absorbance changes identical to those in Figure 1D. When this reaction was repeated using either 0.5 μM CO-myoglobin (holoMb trace) or apomyoglobin denatured in 5 M GdmCl (unfolded trace), only a small slow phase was observed. When the reaction of CO-heme with apomyoglobin was carried out in the presence of 5 μM bovine serum albumin, the full absorbance change was observed, but the binding process became much slower (Figure 2A, ApoMb and BSA trace). In the latter experiment, most of the CO-heme initially binds to albumin since it is present in excess, has a greater surface area per molecule, and appears to have more binding sites. CO-heme then dissociates from the BSA and is taken up irreversibly by apomyoglobin to form MbCO. Almost all of the observed absorbance changes are associated with the latter slow, unimolecular process when excess bovine serum albumin is present.

Reaction of apomyoglobin with excess CO-heme results in slow absorbance changes which are also due to nonspecific binding, in this case to newly formed holoprotein (Figure 2B). As the concentration of CO-heme is increased from 0.5 to 5 μM, additional extremely slow phases appear. In this case nonspecific binding occurs after and in addition to the specific process. The additional heme absorbance changes are due to changes from an aqueous to apolar environment, coordination to surface lysines and histidines, and denaturation of the holoprotein containing excessive amounts of nonspecifically bound heme.

All of these results suggest that the slow phases observed in CO-heme binding experiments represent heme binding to and, sometimes, exchange from nonspecific sites. For screening a larger number of mutants, the reaction of CO-heme with apomyoglobin is best carried out at the lowest possible concentrations of heme and with the protein in excess. The absorbance change associated with 0.25 μM CO-heme is sufficient for analysis, and the use of 0.5 μM apomyoglobin results in a half-time of about 25$-$35 ms, which is easily measured in our stopped-flow apparatus.

Heme Binding to BSA and H93G Apomyoglobin. The affinity of bovine serum albumin for heme is due primarily to nonspecific hydrophobic interactions, although some of the bound heme molecules appear to be coordinated to endogenous bases (Marden *et al*., 1989). The 10^6-fold higher affinity of myoglobin for hemin is due to the specificity of the heme pocket and the strength of the proximal histidine$-$heme iron coordination bond. To estimate the relative importance of each of these two factors, rates of heme binding to and dissociation from bovine serum albumin and H93G myoglobin were measured. The affinity of H93G apomyoglobin for heme is determined solely by noncovalent interactions with heme pocket residues. Even when the heme is coordinated to exogenous imidazole, there is no direct covalent linkage to the protein (Barrick, 1994). As shown in Table 1, each of these proteins has about the same CO-heme association rate constant as apomyoglobin ($k_H' \approx 7 \times 10^7$ M^{-1} s^{-1}), and consequently, their affinities for heme are governed by the dissociation rate constant (k_{-H}).

H93G myoglobin forms a stable complex with hemin in the presence of exogenously added imidazole (Barrick, 1994). The spectral and functional properties of this complex are similar to those of native aquometmyoglobin, and hemin loss can be measured using the H64Y/V68F apomyoglobin assay. The observed rate constant for the later process is $\sim$ 0.04 s^{-1} in the presence of a stoichiometric amount of imidazole. Assuming that k_H' is the same for both CO-heme and monomeric hemin, the affinity of H93G myoglobin for hemin is estimated to be $\sim 6 \times 10^9$ M^{-1}.

The observed rate of hemin dissociation from bovine serum albumin is $\sim$0.01 s^{-1} and very similar to that observed for H93G myoglobin. Assuming an association rate constant of $\sim 5 \times 10^7$ M^{-1} s^{-1}, the affinity of BSA for hemin is also

Table 1: Kinetic and Equilibrium Parameters for Hemin and CO-Heme Binding to Bovine Serum Albumin and to Wild-Type and H93G Myoglobin[a]

protein	k_H' (M^{-1} s^{-1})	k_{-H} (s^{-1})	K_H (M^{-1})
wild-type Mb/hemin	7×10^7 [b]	8.4×10^{-7}	8×10^{13}
H93G Mb/hemin	7×10^7 [b]	1.2×10^{-2}	6×10^9
BSA/hemin	$\sim 5 \times 10^7$ [b]	1.1×10^{-2}	4×10^9
BSA/CO-heme (weakly bound)	5×10^7	7.7	7×10^6

[a] All association rate constants were measured using CO-heme in 50 mM Tris, 50 mM NaCl, pH 8.0 at 20°C . These rate constants are assumed to apply to all monomeric forms of iron-porphyrin including hemin. Hemin dissociation from H93G myoglobin was measured in 0.15 M potassium phosphate, 0.45 M sucrose, pH 7.0 at 37 °C. This k_{-H} value was extrapolated to 20 °C using $E_a = 12$ kcal/mol and is assumed to apply at both pH 7 and 8 (Hargrove *et al*., 1994a). Hemin and CO-heme dissociation from bovine serum albumin (BSA) was measured at pH 7.0, 20 °C. K_H was computed as the ratio k_H'/k_{-H} and is an estimate of hemin affinity for the apomyoglobin samples. [b] These values of k_H' were measured for the binding of CO-heme and are assumed to apply to monomeric hemin binding.

$\sim 5 \times 10^9$ M^{-1}. CO-heme dissociation from albumin involves the dissociation of CO-heme from both the surface of the protein and hydrophobic pockets containing coordinating bases. In experiments not shown, an initial, very rapid process is observed at 423 nm when BSA containing CO-heme is mixed with apomyoglobin. The rate of this phase, ~ 8 s^{-1}, is similar to that seen for the slow phase in the reaction of apomyoglobin with free CO-heme and probably represents the dissociation of nonspecifically bound and presumably 5-coordinate CO-heme. This dissociation rate constant suggests an association equilibrium constant of $\sim 6 \times 10^6$ M^{-1} for nonspecific CO-heme binding to BSA (Table 1).

Effects of Globin Mutagenesis. Point mutations which place a polar residue in the heme pocket destabilize the native state of apomyoglobin (Hargrove *et al.*, 1994b). To determine if the structural changes incurred by these mutations affect the association reaction, rate constants for CO-heme binding to a number of these myoglobin mutants were measured. The values of k_H' were also determined for CO-heme binding to several other native myoglobins, soybean leghemoglobin, and isolated α and β chains of human hemoglobin. The results are shown in Table 2.

None of the proteins listed in Table 2 has a bimolecular rate constant of CO-heme binding deviating more than 4-fold from that of wild-type apomyoglobin, even though the stabilities of the apoproteins vary over 10^6-fold (Hargrove *et al*., 1994b). To a first approximation, each apoglobin binds CO-heme with a second-order rate constant $\approx 1 \times 10^8$ M^{-1} s^{-1}. These mutations cause marked effects on the function of myoglobin, and there are significant differences in structure between myoglobins, leghemoglobin, and hemoglobin chains. The invariance of k_H' suggests strongly that the bimolecular association reaction is driven by a general hydrophobic effect in which water extrudes the apolar heme group into the globin. This conclusion is supported by the experiments of Gibson and Antonini (1963), Chu and Bucci (1979), and Rose and Olson (1983), in which modified CO-hemes reacted with globin at nearly the same rate as CO protoheme. As a result, specificity is manifested primarily by differences in heme dissociation rate constants.

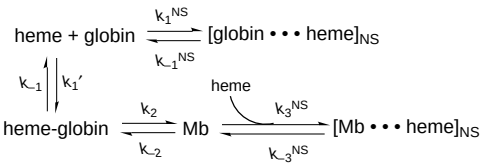
Table 2: Association Rate Constants for CO-Heme Binding to Native Myoglobins, Myoglobin Mutants, and Other Heme Proteins[a]

apoprotein	k_H' (M^{-1} s^{-1}), pH 8.0, 20 °C
(A) Native Heme Proteins	
sperm whale Mb	$(5 \pm 3) \times 10^7$
horse Mb	14×10^7
sheep Mb	14×10^7
soybean LbA	5×10^7
Hb α chains	4×10^7
Hb β chains	7×10^7
(B) Recombinant Proteins	
(1) Sperm Whale Mb	
wild-type	7×10^7
L29F	14×10^7
R45H	16×10^7
R45K	9×10^7
R45T	18×10^7
R45E	4×10^7
R45A	16×10^7
R45S	18×10^7
R45Y	14×10^7
R45D	5×10^7
F46V	11×10^7
H64L	11×10^7
H64Y	6×10^7
G65V	6×10^7
V68A	16×10^7
V68F	7×10^7
V68W	12×10^7
V68Q	4×10^7
V68T	13×10^7
L89G	12×10^7
L89A	17×10^7
L89S	12×10^7
L89F	4×10^7
L89W	12×10^7
H97A	10×10^7
H97V	5×10^7
H97D	6×10^7
I107F	6×10^7
(2) Human Mb	
wild type	21×10^7
H64V/V68H	14×10^7
(3) Pig Mb	
V68T	22×10^7

[a] In most cases, the reactants were 0.50 μM apoglobin and 0.25 μM CO-heme after mixing. The reactions were carried out in 50 mM Tris and 50 mM NaCl at pH 8.0, 20 °C. Buffer was equilibrated with 1 atm of CO, and sodium dithionite was added to prevent oxidation. Each time course was fitted to a two-exponential expression. The rate constants listed apply to the large, bimolecular phase which accounts for 70−90% of the total absorbance change observed in each experiment.

DISCUSSION

Kinetic Mechanism of CO-Heme Binding. Our new data support the basic model proposed by Gibson and Antonini (1960) for describing the reaction of heme with apoglobins. Some modification is required to account for the slow first-order phases that are observed even when monomeric CO-heme is used:



(2)

The super- and subscripts NS refer to nonspecific binding.

In this scheme, heme is rapidly absorbed into apolar regions of the protein. The primary site of binding in apomyoglobin is the heme pocket and rapid formation of the final holoprotein complex occurs when this site is occupied (*i.e.*, $k_2 \geq 500$ s^{-1}). However, some adsorption of heme at nonspecific sites ($[globin\cdots heme]_{NS}$) appears to occur. In the presence of excess apoglobin, nonspecifically bound heme must dissociate before final incorporation into the heme pocket. When excess heme is present, heme binds nonspecifically to the holoprotein ($[Mb\cdots heme]_{NS}$).

This mechanism is supported by four observations: (1) The rate of the slow phase for CO-heme binding is independent of protein concentration when protein is in excess; (2) reactions with excess CO-heme are complex and the amplitudes of the slow phases increase as CO-heme concentration is raised; (3) the initial rates of dissociation of 5-coordinate CO-heme from bovine serum albumin are similar to the rates of the slow phases observed when CO-heme is mixed with excess apomyoglobin; and (4) the estimated equilibrium constants for the initial formation of both the specific (k_1/k_{-1}) and nonspecific (k_1^{NS}/k_{-1}^{NS}) heme−globin complexes are on the order of that observed for pentacoordinate CO-heme binding to albumin, *i.e.*, 10^5-10^7 M^{-1} (Table 1, eq 3).

The final formation of holomyoglobin involves coordination of His^{93} to the iron atom, but this process appears to be governed by larger scale protein conformational transitions. In studies with apohemoglobin, Rose and Olson (1983) observed a large dependence of k_2 on exogenous glycerol concentrations suggesting a substantial dependence on solvent viscosity. Their observations, combined with the results of Leutzinger and Beychok (1981) for heme-induced refolding of α globin, suggest that k_2 is associated with the final folding of the apoglobin around the prosthetic group. This conformational event occurs in a few milliseconds, is slower than the early events in apomyoglobin folding but is similar to or faster than the final formation of native apoprotein measured by Jennings and Wright (1993).

When globin is in excess and in the absence of nonspecific binding, the overall pseudo-first-order rate constant is given by $k_2[Gb]/(K_d + [Gb])$ where $K_d = k_{-1}/k_1'$. At the low protein concentrations required to measure the heme binding reaction by rapid mixing, the observed rate depends linearly on [globin], and the apparent second-order rate constant is given by $k_H' = k_2/K_d$. This observation suggests that K_d is significantly greater than the protein concentration used, *i.e.*, $K_d \geq 1 \times 10^{-5}$ M.

In the case of dicyanohemin binding, the rate-limiting step for holoprotein formation is not folding around the heme group. Instead, a CN^- ligand must dissociate before the proximal histidine can coordinate to the heme iron. The rapid fluorescence quenching observed in Figure 1C shows that dicyanohemin associates with the apoprotein at a rate similar to that observed for CO-heme. However, the major change in Soret absorbance only occurs when the proximal histidine displaces coordinated CN^-.

Problems with Hemin Association. The situation for aquohemin binding is more complex. Adams (1977) and Kawamura-Konishi and Suzuki (1985) examined the reactions of hemin with apomyoglobin and apohemoglobin, respectively, under conditions designed to minimize hemin aggregation. Adams (1977) kept hemin at pH 13 until just before mixing with a strongly buffered solution containing an equimolar concentration of apomyoglobin. He measured a bimolecular association rate constant $= 3.3 \times 10^5$ M^{-1} s^{-1} for hemin binding at pH 7.2. Kawamura-Konishi and Suzuki (1985) used caffeine, which intercalates between porphyrin rings, to solubilize hemin aggregates at neutral pH. They measured a bimolecular association rate constant $= 2.9 \times 10^6$ M^{-1} s^{-1} at pH 7.2 for the reaction of hemin/caffeine with apohemoglobin, but this phase accounted for only a fraction of the total absorbance change.

The initial bimolecular phases observed by Gibson and Antonini (1960) for hemin binding to apohemoglobin at pH 9 and by us for hemin binding to apomyoglobin (Hargrove *et al*., 1994a) are somewhat faster (up to 10^7 M^{-1} s^{-1}), but the absorbance changes associated with these rapid changes are very small and only observed at extremely low hemin concentrations. It is probable that all these measurements underestimate the "true" association rate constant for the binding of monomeric hemin to apoglobin. In the case of Adams (1977), the μ-oxo dimers formed at high pH are likely to react more slowly with apomyoglobin than monomers, even when quickly placed in a neutral pH environment. In the case of Kawamura-Konishi and Suzuki (1985), the reactive species is the hemin/caffeine complex, which is also likely to react more slowly than a simple 5-coordinate aquo complex. Thus, for estimations of hemin affinity and comparisons between mutants, we have tentatively assumed that the association rate constant for monomeric hemin binding to apoglobins is the same as that for CO-heme binding, $\sim 1 \times 10^8$ M^{-1} s^{-1}, and independent of protein structure based on the results in Table 2.

Factors Governing Heme Affinity. The overall association equilibrium constant for hemin binding to apomyoglobin to form metmyoglobin is roughly 10^{14} M^{-1} (Table 1). The equilibrium constant for the initial heme/apoglobin complex is estimated to be 10^5-10^7 M^{-1} based on the affinity of bovine serum albumin for weakly bound CO-heme, and the values of k_1/k_{-1} estimated for both apohemoglobin and apomyoglobin. The driving force for this process appears to be nonspecific partitioning of the amphipathic prosthetic group into apolar regions of the protein. The equilibrium constant for folding around the prosthetic group and Fe^{3+} coordination, k_2/k_{-2}, must be $\sim 10^8$ to account for the overall affinity for hemin.

When the proximal histidine is replaced with Gly, the affinity of the resultant apomyoglobin for hemin decreases from 8×10^{13} to 6×10^9 M^{-1}, suggesting that direct coordination of His^{93} to Fe^{3+} contributes a factor of 10^4 to the overall affinity of wild-type apomyoglobin for hemin. Barrick (unpublished) estimated a similar value of $\sim 10^5$ M^{-1} for the equilibrium constant describing imidazole binding to hemin embedded noncovalently in H93G myoglobin. Thus, under physiological conditions, the individual contributions to the affinity of apomyoglobin for hemin are 10^5-10^7 (~ -30 to -40 kJ/mol) for nonspecific partitioning into the apolar heme pocket, 10^3-10^4 (~ -20 kJ/mol) for formation of the proximal histidine−Fe^{3+} bond, and 10^3-10^4 (~ -20 kJ/mol) for specific interactions with amino acid residues surrounding the porphyrin ring. The strength of the Fe−His^{93} bond is governed by the protein through restrictions in coordination geometry, the electrostatic environment of the proximal histidine, and interactions with the other axial ligand, which is normally a water molecule in ferric myoglobin. It is striking that the majority of the free energy

released by hemin binding is due to simple hydrophobic partitioning. This result accounts for the tendency of heme groups to adsorb nonspecifically to almost all proteins and for the lack of dependence of the association rate constant on exact protein structure.

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