Stability of the Heme-Globin Linkage in \( \alpha \beta \) Dimers and Isolated Chains of Human Hemoglobin

A STUDY OF THE HEME TRANSFER REACTION FROM THE IMMOLIZED PROTEINS TO ALBUMIN*

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The stability of the heme-globin linkage in \( \alpha \beta \) dimers and in the isolated chains of human hemoglobin has been probed by studying the transfer of heme from the proteins immobilized onto CNBr-activated Sepharose 4B to human albumin. The kinetic and equilibrium features of the reaction have been measured spectrophotometrically given the stability of the heme donors and the ease with which heme donor and acceptor can be separated. Isolated \( \alpha \) and \( \beta \) chains transfer heme to albumin at similar rates (1–6 \times 10^{-2} \text{ s}^{-1} \) at pH 9.0 and 20°C in the ferrous CO-bound and in the ferric state. In \( \alpha \beta \) dimers, the heme-globin linkage is strengthened considerably, albeit to a different extent in the ferrous CO-bound and ferric met-aquo derivatives. Only in the latter heme is lost at a measurable rate, 0.065 ± 0.011 \times 10^{-2} \text{ s}^{-1} for \( \alpha \) heme and 2.8 ± 0.6 \times 10^{-2} \text{ s}^{-1} for \( \beta \) heme at pH 9.0 and 20°C, which is very close to the rate measured with soluble met-aquo-hemoglobin at micromolar concentrations. These results indicate that in human hemoglobin the heme-globin linkage in the \( \alpha \) chains is stabilized by interactions between unlike chains at the \( \alpha \beta_1 \) interface, whereas heme binding to the \( \beta \) chains is stabilized by interactions at the \( \alpha \beta_2 \) interface. These long range factors have to be taken into account in addition to the local factors at the heme pocket when evaluating the effect of point mutation and chemical modification.

In the case of human hemoglobin (HbA) there are relatively few quantitative estimates of the stability of the heme-globin linkage despite its relevance in the study of mutant and chemically modified hemoglobins (2–6). The interaction between heme and globin is known to be affected by a number of parameters. Of major importance are the redox state of the protein and pH (2, 4, 5). Thus, heme is released only from oxidized, met-aquo-hemoglobin, but not from the reduced protein and more so upon departure from neutral pH values. In met-aquo-hemoglobin, the rate of heme release from \( \beta \) chains is considerably faster than from \( \alpha \) chains. Furthermore, the kinetics of heme dissociation from \( \alpha \beta \) dimers is much greater than from \( \alpha \beta_2 \) tetramers (5, 6). In the isolated \( \alpha \) and \( \beta \) chains to our knowledge the heme-globin affinity has never been measured, presumably due to the very marked tendency of the respective globins to precipitate. As matter of fact in all studies of heme dissociation from hemoglobin or from myoglobin the formation of free, precipitable globin upon heme depletion of the ferric protein is a common problem (3, 5, 6) that has been alleviated in part by adding sucrose to the incubation medium (2).

We have addressed the problem of globin precipitation in a different manner and propose the use of \( \alpha \beta \) dimers immobilized on Sepharose 4B (7–10) as heme donor. This material offers a number of advantages: it is stable after heme depletion, it does not undergo changes in state of association, it can be lyophilized, and, most importantly, it can be separated easily from the acceptor protein. Therefore formation of the heme-acceptor complex can be followed spectrophotometrically in a facile way under any experimental condition. As heme acceptor we have used human albumin which is endowed with two high affinity heme binding sites (11) and whose affinity for heme does not change in the pH range 5–10 (12). Transfer of heme from hemoglobin to albumin to form methemalbumin is known to occur in the blood of patients with plasma hemoglobinemia and “in vitro” when albumin is mixed with ferric hemoglobin (4, 5). The immobilized \( \alpha \beta \) dimers behave like the soluble protein in that they do not transfer heme to albumin when in the oxy or CO form, but only when in the oxidized state, an indication that the immobilization process does not produce significant alterations in the heme environment. The direct comparison of the rate of heme loss from soluble dimers has confirmed this contention.

The behavior of isolated \( \alpha \) and \( \beta \) chains both in solution and after immobilization on Sepharose 4B has also been studied. Quite unexpectedly, soluble \( \alpha \) and \( \beta \) chains transfer heme readily to albumin even when oxygenated or in the CO-bound form, a finding which points to a major change in the heme pocket induced by the assembly of unlike chains into a heterodimer.
Heme Transfer Experiments with Immobilized \( \alpha \beta \) Dimers—Heme transfer from immobilized \( \alpha \beta \) dimers to albumin can be described by the minimal reaction scheme,

\[
\alpha \beta \text{H} + A \xrightleftharpoons{k_A} \alpha \beta \text{H} + \text{A}
\]

\[
\alpha + \text{H} \xrightleftharpoons{k_a} \alpha \text{H}
\]

\[
\beta + \text{H} \xrightleftharpoons{k_b} \beta \text{H}
\]

\[
\text{Scheme I}
\]

where \( \alpha \beta \) is the immobilized dimer, \( A \) stands for albumin and \( H \) for heme.

In view of the different behavior of \( \alpha \) and \( \beta \) chains, the kinetic analysis was carried out by approximating the overall reaction to the following equilibria,

\[
\alpha \beta \text{H} + A \xrightleftharpoons{k_A} \alpha \beta \text{H} + \text{A}
\]

\[
\alpha + \text{H} \xrightleftharpoons{k_a} \alpha \text{H}
\]

\[
\beta + \text{H} \xrightleftharpoons{k_b} \beta \text{H}
\]

\[
\text{Scheme I}
\]

where \( \alpha \) and \( \beta \) are the hemoglobin chains in the immobilized \( \alpha \beta \) dimer.

An iterative diagonalization of the rate constants matrix relative to the processes described by the equilibria (Equations 1–3) was used to fit the time course of methemalbumin formation. The second order processes were linearized by assuming \( dh/dt = 0 \). In the fitting procedure: (i) the rate constants \( k_a \) and \( k_a \) were fixed at the values determined in independent experiments, i.e. at \( 5 \times 10^{9} \text{ M}^{-1} \text{s}^{-1} \) and \( 3.2 \times 10^{9} \text{ M}^{-1} \text{s}^{-1} \); (ii) the concentrations of heme-depleted \( \alpha \) and \( \beta \) chains as well as the dissociation rate constants \( k_{1a} \) and \( k_{1b} \) were allowed to float; (iii) the amount of heme associated with the globin chains and with albumin was fixed to the value determined experimentally at the end of the reaction (see Fig. 8), thereby fixing the values of the rates for heme binding, \( k_a \) and \( k_b \), at each iteration.

Heme Transfer Experiments with Soluble Hemoglobin—In these experiments human hemoglobin after oxidation with an excess of potassium ferricyanide was equilibrated with 0.1 M Tris-HCl plus 0.1 M NaCl and 0.3 M sucrose, pH 9.0, on a Sephadex G25 column. Hemoglobin solutions at \( 5 \times 10^{-5} \text{ M} \) (in heme) were mixed with the same volume of \( 5 \times 10^{-5} \text{ M} \) albumin in the same buffer and the heme transfer reaction was followed at 410 nm in a Hewlett-Packard 8542A spectrophotometer for about 180 min at 20°C. The kinetics of heme transfer was analyzed in terms of three first order processes corresponding to: 1) heme release from the \( \beta \) chains in \( \alpha \beta \) dimers, 2) heme release from \( \beta \) chains in \( \alpha \beta \beta \) trimers, 3) a slow process which comprises heme release from the \( \alpha \) chains and heme release from methemalbumin. In the fitting procedure the relative amplitudes of processes 1 and 2 were fixed at the amounts of dimers and trimers present at equilibrium on the basis of the dimmer-tetramer association constant measured in parallel ultracentrifugation experiments. The amplitude of the slow phase and the three rate constants were allowed to float.

Reaction of Albumin with Soluble Hemin—Titration experiments were carried out at a constant albumin concentration of \( 1.3 \mu \text{M} \) in \( 0.1 \text{ M} \) Tris-HCl plus 0.1 M NaCl, pH 9.0, by adding small volumes of a hemin solution in the same buffer at 20°C. Given the low concentrations involved a 10 cm column was used. Formation of the methemalbumin complex was followed at 403 nm. At any given point in the titration the contribution of free hemin to the observed absorbance was subtracted. The data were analyzed according to a simple two sites Adair equation,

\[
Y = \frac{K_1[H] + 2K_2[H]^2}{K_1[H] + K_2[H]^2} + \frac{1}{K_3[H] + K_4[H]^2} + K + \text{c}
\]

\[
\text{Eq. 4}
\]

The abbreviation used is: bis-Tris, 2-[bis(2-hydroxyethyl) amino]-2-(hydroxymethyl)propane-1,3-diol.
Heme Dissociation From αβ Dimers and Hemoglobin Chains

RESULTS

Heme Binding to Albumin—The reaction between heme and albumin has been reinvestigated prior to performing the heme transfer experiments. The titration curve of albumin with heme (Fig. 2) was analyzed according to Equation 4 and points to the presence of two high affinity binding sites for heme, characterized by $K_1 = 7.8 \times 10^7 \text{M}^{-1}$ and $K_2 = 2.3 \times 10^8 \text{M}^{-1}$ in good agreement with the values reported in Ref. 11.

The kinetics of the reaction has been investigated by mixing in a stopped flow apparatus 3.6 μM heme with albumin at concentrations varying between about 20 and 250 μM. Hemin was used at a low concentration to minimize its tendency to polymerize. The time course is monophasic and has been analyzed as a first order reaction. The pseudo first order rate constant (Fig. 3) depends linearly on heme concentration up to approximately 25 μM; at higher protein concentrations a constant value of 1.3 s$^{-1}$ is reached. This value, which relates to a rate-limiting monomolecular step, is close to the rate of heme depolymerization (18). From the data in Fig. 3 an association rate constant for heme binding to albumin of 5 (±0.9) $\times 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$ can be obtained. This value in combination with the average affinity constant determined independently (see above) yields an average rate of heme dissociation from albumin of 3.2 (±0.11) $\times 10^{-4} \text{s}^{-1}$.

Heme Transfer Experiments from α and β Chains to Albumin—Either type of chain releases heme to albumin at pH 9.0 not only when oxidized, but also when in the CO-ligated state. The shift of a substantial amount of heme from CO-bound α and β chains to albumin is clearly shown by the electrophoresis patterns of Fig. 4. Under similar experimental conditions, there is no heme transfer from CO-ligated HbA to albumin as reported by Bunn and J and (4).

Immmobilized CO-ligated α and β chains exposed to albumin at pH 9.0 and 20°C behave similarly to the soluble ones and give rise readily to a significant amount of methemalbumin (Fig. 5A). It is of interest that methemalbumin is formed indicating that CO is lost upon or before binding. An equilibrium distribution is attained after about 180 min; at equilibrium, when albumin is in 3-fold molar excess (in terms of heme binding sites), about 50% of the heme is in the form of methemalbumin. Under similar experimental conditions immobilized oxidized chains loose all their heme (Fig. 5A).

The heme transfer process displays two clearly separated kinetic phases (Fig. 6). The time course was fitted using the scheme and the approximations presented under "Materials and Methods" by considering the contribution of only one chain. The rate of the slow phase was taken as $3.2 \times 10^{-4} \text{s}^{-1}$, which corresponds to the rate of heme dissociation from albumin estimated from the measurements on the heme-albumin system. The rate of the fast phase, which can be assigned to heme release from the α or β chains is $4.5-7.5 \times 10^{-2} \text{s}^{-1}$ and does not depend within experimental error on the chain type and on the state of heme oxidation (Table I).

In a further set of experiments the observation that methemalbumin is formed upon transfer of heme from the CO-bound chains was exploited as it enables measurement of heme transfer from soluble chains to albumin. Upon mixing β chains at 7
3.4 M albumin at pH 9 and 20°C in the presence of 0.3 M sucrose (2). A biphasic reaction is observed (Fig. 7). The fast rate, 0.2 × 10^{-2} s^{-1}, is roughly 5-fold slower than that measured with immobilized β chains.

Heme Transfer Experiments from Immobilized αβ Dimers to Albumin—It was first established that immobilized αβ dimers, like soluble hemoglobin, do not transfer heme to human albumin when in the oxy, CO, or CN-met form, but dissociate heme rapidly in the ferric, met-aquo form (Fig. 5B). This finding is of importance as it shows that the immobilization and lyophilization steps do not produce significant alterations in the heme environment.

Thereafter heme transfer experiments were carried out by mixing different amounts of immobilized met-aquo αβ dimers with a constant concentration of albumin at pH 9.0 and incubating the mixture at 20°C. Under these experimental conditions an equilibrium distribution is reached within 24 h. The heme transferred from the immobilized dimers to albumin at equilibrium is around 30% when heme is in excess over albumin and approaches 100% at the lowest heme/albumin ratio tested (Fig. 8); conversely, the saturation of albumin with heme increases with increase in heme/albumin molar ratio (data not shown).

The time course of methemalbumin formation at different heme/albumin molar ratios is given in Fig. 9A. It can be described by a fast and a slow process whose apparent rate differs by approximately 50-fold, both rates increase slightly upon increasing pH from 6.5 to 9.0 (see Table I). The amplitude of the

\[
\begin{array}{ccc}
\text{Protein} & k_{α} \times 10^2 & k_{β} \times 10^2 \\
αβ ferric, pH 9.0 & 0.065 \pm 0.011 & 2.8 \pm 0.6 \\
αβ ferric, pH 9.0^a & 1.1 & 0.2 \\
αβ ferric, pH 9.0^a & 0.055 \pm 0.011 & 1.7 \pm 0.8 \\
αβ ferric, pH 6.5 & 0.047 \pm 0.013 & 1.3 \pm 0.9 \\
α ferric, pH 9.0 & 6.3 \pm 1.9 & 4.1 \pm 1.5 \\
β ferric, pH 9.0 & 3.4 \pm 1.2 & 1.2 \pm 0.7 \\
α CO, pH 9.0 & 0.2 \pm 0.08 \\
α CO, pH 9.0 & 0.2 \pm 0.08 \\
\end{array}
\]

\(^a\) Data in solution.
fast process predominates at the higher heme/albumin molar ratios (Table II).

Bunn and Jandl (4), when studying the exchange of hemes between hemoglobins A and F, observed that the process is biphasic and proposed that the fast rate reflects the dissociation of heme from non-α chains and the slower one dissociation from the α chains. This assignment has been confirmed recently by Hargrove et al. (2) with the aid of mutant hybrid methemoglobins and valence hybrids in which one subunit is oxidized.

The data in Fig. 9 can be interpreted in the same way: when hemoglobin is in excess, rapid dissociation of heme from the β chains predominates as indicated by the greater amplitude of the fast process; when albumin is in excess, the contribution of heme dissociation from the α chains becomes relevant and the amplitudes of the fast and slow process are approximately equal. Under all conditions, as the reaction proceeds, there is an accumulation of the heme-albumin complex, and thus, a significant contribution of the rate of hemin dissociation from albumin, in particular to the slow phase. This interpretation was substantiated by the analysis of the heme transfer reaction carried out as outlined under "Materials and Methods." Both rate constants pertaining to albumin, $k_A$ and $k_{\text{A}}$, $5 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $3.2 \times 10^{-4} \text{ s}^{-1}$, respectively, were calculated using the data of Figs. 2 and 3. Likewise, the equilibrium partitioning of hemin between albumin and the immobilized $\alpha\beta$ dimers was fixed at the value determined at the end of the experiment reported in Fig. 8. The global fit of the whole set of data yields rate constants of $6.5 \times 10^{-4}$ and $2.8 \times 10^{-2} \text{ s}^{-1}$, respectively, for the slow and fast phase; the fitted time courses are shown in Fig. 9.

Heme transfer experiments similar to those just described were carried out also at pH 7.5 and 6.5. The amount of heme transferred to albumin at equilibrium increases at any given
The amount of tetramers present in solutions of methemoglobin is 0.008 and the value of the offset 0.261. The scheme given under "Materials and Methods." The amplitude of the slow phase is in very good agreement with that from soluble dimers. To this end it appeared necessary to determine the dimer-tetramer association constant of 1.1 × 10^6 M^-1 (dimer basis).

pH with decrease in the heme/albumin molar ratio; at any given ratio of albumin to heme it decreases with decrease in pH. The rate constants which describe the time course of heme exchange are affected only slightly by pH (Table I). Representative fits at pH 7.5 are included in Fig. 9.

Last a set of heme transfer experiments from soluble hemoglobin was performed at pH 9.0 and 20 °C. These measurements were designed to compare under the same conditions of pH and temperature the rate of heme release from immobilized dimers with that from soluble dimers. To this end it appeared necessary to determine the dimer-tetramer association constant of methemoglobin by ultracentrifugation. To our knowledge there are no reports in the literature on this equilibrium.

Under the conditions used for the heme transfer experiments, namely Tris buffer 1 = 0.1 M + 0.1 M NaCl at pH 9.0 and 20 °C, the dimer-tetramer association constant was found to lie between 3.2 × 10^4 and 8.0 × 10^4 M^-1 (dimer basis) by sedimentation velocity (Fig. 10). Preliminary sedimentation equilibrium experiments carried out at 10 °C yielded 5 × 10^4 M^-1. In view of the small temperature dependence of the dimerization reaction at alkaline pH values (ΔH^a,2 = 13.2 ± 2 kcal/mol; Ref. 19), the K^a,2 value corrected to 20 °C, 9 × 10^4 M^-1, is close to the upper limit obtained by sedimentation velocity. Thus, when the concentration of soluble methemoglobin is 2.5 × 10^-6 M (heme), the fraction of dimers is close to 90%. The fit to the heme transfer data at this hemoglobin concentration, carried out as described under "Materials and Methods," yields rates of 1.1 × 10^-2 s^-1 and 0.2 × 10^-2 s^-1 for heme loss from β chains in dimers and tetramers, respectively, and 3.8 × 10^-4 s^-1 for the slow process (Fig. 10). The rate of heme release from β chains in soluble dimers is in very good agreement with that from immobilized ones (Table I). Likewise the rate of the slow process is fully consistent with the values obtained for heme loss from chains in the dimer (Table I) and from methemalbumin.

Sedimentation velocity experiments were also carried out in 0.15 M phosphate buffer, pH 7.0 and 20 °C, in order to assess the amount of tetramers present in solutions of methemoglobin under conditions similar to those used by Hargrove et al. (2). Dissociation into dimers is less pronounced than in Tris buffer at pH 9.0 (K^a,2 = 1.5 × 10^5 M^-1). This finding is not unexpected since dissociation into dimers of oxyhemoglobin, which entails cleavage of the same αβ interface, is likewise enhanced at alkaline pH values (20).

**DISCUSSION**

The experiments presented here show that the stability of the heme-globin linkage in αβ dimers and isolated chains of human hemoglobin can be probed by studying the heme transfer reaction from the immobilized proteins to albumin. Soluble and Sepharose-bound heme-proteins transfer heme to albumin in a similar fashion. From a qualitative viewpoint, only those derivatives which transfer heme to albumin in solution do so when immobilized, and conversely, those derivatives which do not release heme to albumin in solution do not release heme when bound to Sepharose (Fig. 5, A and B). At a quantitative level the direct comparison carried out with both αβ dimers and isolated chains indicates that the immobilization step does not alter the heme environment significantly while providing material that is not easily denatured after heme loss. Thus, the rates of heme transfer from the immobilized proteins are increased only 3- to 6-fold relative to the soluble ones (Table I).

Hemoglobin donors have other advantages; for example they do not undergo changes in state of association, which may complicate analysis of the heme transfer reaction in solution, and can be separated easily from the heme acceptor. The latter property enables one to monitor the relevant spectral changes easily even when the spectral properties of heme donor and acceptor are very similar. In brief, immobilized heme donors provide a solution to several problems that have limited the study of the heme transfer reaction in solution (2, 5, 6).

In the present work human albumin has been used as the heme acceptor, because its affinity for heme is comparable with that of human hemoglobin (11). This feature permits determination not only of the kinetic, but also of the equilibrium aspects of the heme transfer reaction. In turn, knowledge of the amount of heme partitioned at transfer equilibrium between the hemoprotein and albumin provides a useful constraint in the fit of the time courses to the reaction scheme.

The most interesting finding of this study is that the stability of the heme-globin linkage changes dramatically upon formation of αβ dimers. This change is especially striking in the ferrous CO-bound state at pH 9.0 and 20 °C the isolated chains transfer heme significantly to albumin over a time scale of a few hours, whereas no heme release takes place from αβ dimers (Figs. 4 and 5), which behave like the αβ tetramer (4). The assembly of unlike chains, therefore, brings about a major rearrangement in the heme pocket of the ferrous protein which results in considerable strengthening of the heme-globin interaction, such that in practice heme dissociation cannot be measured.

Heme oxidation enhances heme release in human hemoglobin (2, 4, 5) and does so also in the αβ dimers (Fig. 5B), but has a very small effect on the heme transfer properties of the isolated chains (Table I). This difference in behavior can be ascribed to the difference in the nature of the ferric forms of the proteins. The isolated chains give rise to six-coordinate low spin hemichromes, in which the iron-proximal histidine bond is effectively covalent, while HbA and its immobilized dimers form high spin met-aquo derivatives in which this bond is weakened. It is of interest that isolated α and β chains lose their heme at approximately the same rate (1-6 × 10^-2 s^-1), whereas in the αβ dimer heme dissociation from the α chains is some 50 times slower than from the β chains (e.g. 0.065 × 10^-2 s^-1 versus 2.8 × 10^-2 s^-1 at pH 9.0 and 20 °C). In turn release
of heme from the β chains in the αβ dimer is only 2–3-fold slower than from isolated β chains. The interaction between unlike chains, therefore, enhances the stability of the heme-globin linkage significantly in the α chains and very little in the β chains. It may be envisaged that the constraint on the flexibility of the α chains imposed by the β chains (21) increases the activation energy for α-heme release and that this constraint is transmitted to the α-heme pocket through the αββ2 interface, since immobilized dimers like the soluble ones are of the αββ2 type (7–10). The rate of β-heme release increases with decrease in HbA concentration (6), indicating that dissociation of the αββ2 tetramer, and hence a change at the αββ2 interface, affects the stability of the heme-globin bond in the β chains. Our data (Fig. 10) suggest that α chains are little affected.

Benesch and Kwong (6) report rates of heme loss from β chains in HbA dimers and tetramers under pH and temperature conditions very similar to those used in the present work; release from the α chains was not determined as only 20% of the reaction was followed. The values given by Benesch and Kwong (6) are 5-fold lower relative to those reported in Table I; however, the difference would be less if heme release from albumin had been taken into account. Hargrove et al. (2) report rates of 1.7 ± 1.1 × 10^{-4} s^{-1} for α-heme dissociation and 2.2 ± 0.6 × 10^{-3} s^{-1} for the β-heme at pH 7.0, 37°C, 2–4 μM heme, a roughly 2-fold increase in both rates at pH 8.0 and relatively small temperature effects. These rates are lower than those reported in Table I for α and β chains. However, if one takes the pH dependence into account and the fact that the rate of heme loss from the β chains is decreased by the presence of tetramers (10–25% at 2–4 μM heme under the conditions used by Hargrove et al., 1994), the agreement between the two sets of data is quite good.

Last, a comment on the dimer-tetramer association constant of met-HbA is in order, since the value obtained by sedimentation velocity and equilibrium in the present work is considerably lower than that reported recently by Benesch and Kwong (6). The ultracentrifugation measurements carried out as a function of met-HbA concentration at pH 9.0 yield values of K_{αβ} in the range 3–9 × 10^{10} M^{-1}, whereas Benesch and Kwong (6) obtained 1.4 × 10^{10} M^{-1} from measurements of the initial rate of heme transfer to serum albumin at different hemoglobin concentrations. However, the following considerations render this indirect method questionable. The curve given in Fig. 1, which was used to calculate the dissociation constant for HbA, is not symmetrical as expected on the basis of mass law considerations, indicating that factors other than dissociation into dimers influence the heme loss assay as used by Benesch and Kwong (6). Accordingly, in a graph like that presented in Fig. 2 of the same reference, the data points do not lie on a straight line (in Ref. 6 the data below zero are not reported). Furthermore, Benesch and Kwong (6) take the concordance of the K_{αβ} value assessed by means of the heme loss assay for met-HbA at pH 9.0 with that obtained by Turner et al. (22) for oxy-HbA at pH 7.4 as a suggestion that neither pH nor the oxidation state of the heme iron can have an important effect on the dimer-tetramer equilibrium. This conclusion is in contrast with current knowledge on the subunit dissociation behavior of hemoglobin (20, 22). A wealth of studies carried out on ferrous HbA have shown subunit assembly to be affected by heme and non-heme ligands, by buffer composition and pH. For instance, alkaline pH values favor dissociation, and the sensitivity to heme ligands is such that dissociation into dimers of oxy- and CO-HbA differs significantly. Oxidation is therefore expected to affect subunit dissociation and indeed the present sedimentation data show that it enhances dissociation. Moreover, dissociation of met-HbA can be expected to be influenced by the same factors which influence the stability of the ferrous tetramer, since the same subunit interface is involved.

In conclusion, the present data bring out that in hemoglobin the heme-globin linkage in the α chains is stabilized by interactions between unlike chains at the αββ2 interface, whereas binding to the β chains is stabilized by interactions at the αββ2 interface. In accordance with this conclusion in mutant hemoglobins with point mutations at the αββ2 interface, the rate of heme release from the β subunits is increased with respect to HbA (3). No data on the effect of mutations at the αββ2 interface are available. In addition to these long range effects, which have to be taken into account in any systematic analysis of heme dissociation in mutant and chemically modified hemoglobins, local factors at the heme pocket play an important role in the stabilization of the heme-globin bond. These comprise steric contacts of the porphyrin ring and electrostatic interactions of its propionate with specific side chains in the distal heme pocket like His-64, Val-68, Ser-92, and Arg 45, as discussed by Hargrove et al. (2) in their recent study on myoglobin and hemoglobin hybrids. In the understanding of the complex interplay between all these factors in determining the stability of the heme-globin linkage, the use of immobilized heme donors and of high affinity heme acceptors, which are in preparation, will be of value.

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