Quaternary Structure Regulates Hemin Dissociation from Human Hemoglobin

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Rate constants for hemin dissociation from the α and β subunits of native and recombinant human hemoglobins were measured as a function of protein concentration at pH 7.0, 37 °C, using H64Y/V68F apomyoglobin as a hemin acceptor reagent. Hemin dissociation rates were also measured for native isolated α and β chains and for recombinant hemoglobin tetramers stabilized by α subunit fusion. The rate constant for hemin dissociation from β subunits in native hemoglobin increases from 1.5 h⁻¹ in tetramers at high protein concentration to 15 h⁻¹ in dimers at low concentrations. The rate of hemin dissociation from α subunits in native hemoglobin is significantly smaller (0.3–0.6 h⁻¹) and shows little dependence on protein concentration. Recombinant hemoglobins containing a fused di-α subunit remain tetrameric under all concentrations and show rates of hemin loss similar to those observed for wild-type and native hemoglobin at high protein concentration. Rates of hemin dissociation from monomeric α and β chains are much greater, 12 and 40 h⁻¹, respectively, at pH 7, 37 °C. Aggregation of monomers to form αβ dimers greatly stabilizes bound hemin in α chains, decreasing its rate of hemin loss 20-fold. In contrast, dimer formation has little stabilizing effect on hemin binding to β subunits. A significant reduction in the rate of hemin loss from β subunits does occur after formation of the αβ interface in tetrameric hemoglobin. These results suggest that native human hemoglobin may have evolved to lose heme rapidly after red cell lysis, allowing the prosthetic group to be removed by serum albumin and apohemoglobin.

Hemoglobin is released by a small amount of red cell lysis, it is diluted significantly in plasma. In the case of human hemoglobin, this dilution leads to formation of noncooperative, αβ dimers which display epitopes that are recognized by circulating haptoglobin molecules (2, 3). Binding of dimers to haptoglobin facilitates rapid clearance from the blood stream (Fig. 1). Hemoglobin dimers also autooxidize more rapidly and lose heme more readily than hemoglobin tetramers (4–6). Rapid heme loss from dilute, extracellular hemoglobin may be advantageous since the resulting free heme in plasma is readily taken up by serum albumin and apohemoglobin and transported to the liver for recycling. Thus, we felt that it would be important to measure quantitatively how the state of aggregation affects the rate of hemin loss from the α and β subunits of human hemoglobin.

Roughly 30 years ago, Bunn and Jandl (7) measured time courses for ⁵⁹Fe-labeled hemin exchange between human adult and fetal hemoglobins and between adult hemoglobin and human serum albumin. Quantitative analysis suggested that the rate of hemin exchange with β subunits was 5–10-fold greater than with α subunits. Benesch and Kwong (5) showed that partial hemin exchange between methemoglobin and human serum albumin can be followed spectrophotometrically at pH values ≥8. They measured rates of hemin dissociation from β subunits in a variety of native and mutant human hemoglobins. However, even at extremely high concentrations, human serum albumin is unable to extract significant amounts of hemin from α subunits within intact hemoglobin. To overcome this problem, we developed a genetically engineered apoglobin for use as a colorimetric reagent to measure complete time courses of hemin dissociation from both myoglobinins and hemoglobinins (8).

The reagent is a myoglobin mutant in which the distal histidine (His-64) was replaced with tyrosine. The phenolate side chain coordinates to the iron atom giving the ferric form of the mutant holoprotein a “green” color and an absorbance spectrum very different from those of native metmyoglobins and methemoglobinins which appear “brown.” In addition, Val-68(E11) was replaced with Phe to enhance the stability of the apoprotein and to increase its affinity for hemin. When methemoglobin A₉ is mixed with an excess of the H64Y/V68F apo-myoglobin reagent, complete hemin exchange occurs and the observed time course is markedly biphasic (see Fig. 2). Using valence and mutant hybrid hemoglobinins, we were able to confirm that the faster phase represents hemin loss from ferric β subunits and the slower phase hemin loss from α subunits (8).

Our rate constants for hemin loss, which were measured at low hemoglobin concentrations (1–10 μM), were significantly larger than those reported by Bunn and Jandl (7), which were measured at high protein concentration. We speculated that the differences were due to more rapid hemin dissociation from methemoglobin dimers that were present at the low concentra-
tions used in our experiments. Benesch and Kwong (6) confirmed this idea directly by measuring the dependence of the rate of hemin dissociation from β subunits on hemoglobin concentration. However, since Benesch and Kwong (6) were using the human serum albumin assay, they were unable to examine the effects of dimer formation on hemin dissociation from α subunits.

In this work, we have measured the rate constants for hemin loss from isolated α and β subunits, αβ dimers, native tetramers, and recombinant tetramers stabilized by a gene fusion. The rate constants for hemin dissociation from native dimers and tetramers were obtained by analyzing the protein concentration dependence of the observed time courses. The results provide a quantitative description of the linkage between quaternary structure and hemin binding in the α and β subunits. These data also explain why it is so difficult to prepare the aquomet forms of isolated chains. Finally, the high rates of hemin dissociation observed in dimers and monomers support the view that human hemoglobin has evolved to fall apart rapidly in dilute solution.

MATERIALS AND METHODS

Preparation of Proteins—A description of the properties of the H64Y/V66F apomyoglobin reagent and its use in measuring complete time courses of hemin loss from ferric myoglobin and hemoglobins are given in Hargrove et al. (8). Native human hemoglobin was purified as described by Mathews et al. (9), and isolated α and β subunits were purified by the method of Bucci (10). Recombinant wild-type human hemoglobin containing V1M replacements in the N terminus of one subunit and αβ,αβ dimers, native tetramers, and recombinant tetramers stabilized by a linker were equilibrated and eluted as described for columns Q2 and S1 in Looker et al. (11). In the present work, the Q-column chromatographic step was repeated after the S-column to remove additional methemoglobin. After the second Q-column procedure, ferrous hemoglobin containing fractions were pooled as above, concentrated to 50 mg/mL and stored as small aliquots in liquid nitrogen.

Measurement of Hemin Dissociation—The general procedures for measurement of hemin dissociation are described in Hargrove et al. (8). Unless otherwise indicated, experiments were performed at 37 °C in 0.15 M potassium or sodium phosphate buffer, pH 7.0 and 0.45 M sucrose. In all experiments, the concentration of apomyoglobin (H64Y/V66F) was at least twice the total Hb (heme) concentration. Under these conditions, the observed rate is equal to the first order rate of hemin dissociation from methemoglobin (8).

Below 12 μM Hb, dissociation time courses were monitored at 410 nm. Between 12 and 35 μM Hb, time courses were monitored at 600 nm. It was not possible to record continuous time courses above 35 μM Hb due to turbidity caused by precipitation of large amounts of apohemoglobin. Consequently, at high hemoglobin concentrations (200–600 μM) heme small aliquots were withdrawn at appropriate time points from a stock reaction mixture and centrifuged briefly (~30 s) at 14,000 rpm in a refrigerated bench top microcentrifuge. A measured volume of supernatant was then diluted into 10 mM sodium phosphate buffer, pH 8, and the visible spectrum recorded quickly. The changes in absorbance at 410 and 600 nm were exported into data analysis software and analyzed as the sum of two independent exponential decay processes. In the latter stages of the reactions (>4 h), the absorbance traces were frequently distorted for samples at initial Hb concentrations greater than 20 μM due to precipitation of apohemoglobin. In these cases the most useful method of analysis was to truncate the absorbance trace to avoid regions showing absorbance increases due to apoprotein aggregation.

RESULTS

Results for Native Human Hemoglobin—Time courses for complete hemin loss from hemoglobin A, are biphasic under all conditions. As shown in Fig. 2, addition of inositol hexaphosphate at low protein concentrations preferentially decreases the rate of the fast or β subunit phase of the reaction from ~16 h⁻¹ to ~3 h⁻¹. The rate constant for the slow phase was unaffected and remained at ~0.5 h⁻¹. This result suggests that tetramer formation stabilizes hemin in β subunits since inosi-
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Thus, the observed rate of hemin loss from an a constants for hemin dissociation are for R-state forms of hemoglobin (3, 15, 16), whereas the rate served for s for hemin dissociation in dimers and in tetramers.

The rate of heme concentration whereas the rate of the slow or aggregation are 1–10 s−1 and the rate of formation and dissociation of hemoglobin little change (Fig. 4). Analysis of these data is simplified by the fact that the rate of formation and dissociation of hemoglobin tetramers is very rapid compared with that for hemin dissociation.

The rate constants for tetramer dissociation and dimer aggregation are 1–10 s−1 and 1–5 × 105 M−1 s−1, respectively, for R-state forms of hemoglobin (3, 15, 16), whereas the rate constants for hemin dissociation are ~0.005 s−1 (Table I). Thus, the observed rate of hemin loss from an a or b subunit is a weighted sum of the rate constants that apply for the subunit in dimers and in tetramers.

\[
k_{obs} = k_{\text{dimer}} + k_{\text{tetramer}}(1 - \gamma_{\text{dimer}})
\]

Eq. 1

where \( \gamma_{\text{dimer}} \) is the fraction of heme groups that are present in dimers. \( \gamma_{\text{dimer}} \) is given by (6, 17)

\[
\gamma_{\text{dimer}} = \frac{K_{\text{dimer}}}{K_{\text{dimer}} + \frac{[Hb]_4}{[Hb]}}
\]

Eq. 2

where \( K_{\text{dimer}} \) is the equilibrium dissociation constant describing the dissociation of hemoglobin tetramers into dimers (\( K_{\text{dimer}} = [Hb]_4/Hb_Hb \)) and \( [Hb] \) is the total concentration of heme groups (\( [Hb] = 2[Hb]_2 + 4[Hb]_4 \)).

The solid line in Fig. 4A for b subunits within native hemoglobin was measured in 0.2 M Bis-Tris, 0.45 M sucrose, pH 7.0, ± 0.2 mM inositol hexaphosphate. The rate of hemin dissociation from b subunits was 16 h−1 in the absence of inositol hexaphosphate and 3 h−1 in the presence of 0.2 mM inositol hexaphosphate. The decrease in \( k_{\text{dimer}} \) for b subunits is due to inositol hexaphosphate-induced aggregation to tetramers.

Effects of inositol hexaphosphate on rates of hemin dissociation from native hemoglobin by Edelstein et al. (17) using flash photolysis and ultracentrifugation techniques. Convergence was more difficult when all three parameters were varied. Similar values of \( k_{\text{dimer}} \) and \( k_{\text{tetramer}} \) were obtained when \( K_{\text{dimer}} \) was fixed to 1 μM (17). Regardless of the exact analysis, the results in Fig. 4A show that \( k_{\text{dimer}} \) for b subunits decreases greater than 10-fold when α1β1 dimers aggregate to form tetramers in agreement with the previous work of Benesch and Kwong (6).

In contrast, the rate of hemin dissociation from a subunits shows little dependence on hemoglobin concentration. If \( K_{\text{dimer}} \) is fixed at 1.5 μM, the fitted values of \( k_{\text{dimer}} \) for a subunits are 0.6 h−1 in dimers and 0.3 h−1 in tetramers. Fits of similar quality were obtained by assuming no dependence and an average value of 0.4 h−1. Regardless of the exact analysis, the results show that dissociation into dimers has little effect on hemin affinity in a subunits.

Recombinant Hemoglobins Stabilized by a Subunit Fusion—Fig. 3B shows time courses for hemin dissociation from recombinant, wild-type human hemoglobin (rHb0.0) and a recombinant human hemoglobin stabilized against dimer formation by fusion of two a subunit genes into a single gene (rHb0.1). In both genes, the codons for the N-terminal valines in both subunits were replaced with methionine codons to initiate translation in E. coli. Since the initiator methionine is retained, each subunit has effectively a V1M mutation (11). In rHb0.1, a glycine residue connects the C terminus of one a chain with the N terminus of a second a chain. This subunit fusion prevents dissociation of the (α-α)β2 tetramer into dimers (11).

Although both proteins were at low concentrations (5–10

![Fig. 2. Effects of inositol hexaphosphate on rates of hemin dissociation from hemoglobin.](image1)

![Fig. 3. A, hemin dissociation from 1 and 600 μM native hemoglobin. The open circles represent data at high concentration and the small dots, data at 1 μM hemoglobin. B, hemin dissociation from 10 μM recombinant human hemoglobin (rHb(0.0)) and 5 μM genetically cross-linked human hemoglobin (rHb(0.1)). Each curve was fitted to a two exponential expression which was forced to have equal amplitudes. Genetic stabilization of hemoglobin tetramers clearly lowers the rate of hemin dissociation from β subunits.](image2)
Hemoglobin, the rate of hemin loss from α subunits appears to be independent of protein concentration and is unaffected by genetic fusion (Fig. 4, Table I).

We examined rates of hemin dissociation from rHb1.1 which, in addition to the V1M mutations and fused α subunits, contains the Presbyterian mutation (β N108K). The latter substitution was added to enhance the O₂ transport properties of the recombinant protein (11). As with rHb0.1, no dependence on protein concentration was observed, and the only difference was a small increase in $k_{-H}$ for β subunits which is presumably due to the N108K mutation in this subunit.

**Hemoglobin from α and β Monomers—**Time courses for hemin dissociation from native isolated α and β chains were measured in a stopped flow apparatus because of their high rates of hemin loss and the difficulty of sample preparation. A slight excess of potassium ferricyanide was added to a syringe containing 20 μM of the oxygenated forms of either α or β chains to generate the corresponding ferric forms. Immediately after formation of the ferric subunit, the contents of this syringe were reacted with 40 μM H64Y/V68F apomyoglobin. Time courses for hemin dissociation were measured at 600 nm to avoid background absorbance by excess ferricyanide ($λ_{max}$ = 400 nm). Slow absorbance increases were observed at the end of each reaction due to precipitation of the newly generated apoglobin chains. Hemin dissociation rate constants were estimated from the initial portions of the time courses by fitting to one or two exponential expressions with an offset (Fig. 5). Hemin dissociation from α chains appears to be monophasic with a rate constant equal to $~12$ h⁻¹ at 37 °C, pH 7. Time courses for hemin dissociation from isolated β subunits were biphasic, with observed rate constants equal to $~40$ h⁻¹ and $~2$ h⁻¹ for the fast and slow phases. McGovern et al. (18) estimated that the equilibrium constant for dissociation of β tetramers into monomers is $1.25 \times 10^{-12}$ M⁻³ at pH 7. This value of $K_{eq}$ predicts a significant amount of β tetramers at the concentrations used in our experiments. Thus, the simplest interpretation of the isolated β chain time course is that hemin dissociation from monomeric β chains is very rapid ($k_{-H} \approx 40$ h⁻¹), whereas hemin loss from β tetramers is slow ($k_{-H} \approx 2$ h⁻¹) and comparable to that from β subunits within tetrameric hemoglobin.

**DISCUSSION**

The quaternary structure of methemoglobin has a profound effect on the rate of hemin dissociation (Table I). The monomeric forms of the isolated α and β chains lose hemin 30–40 times more rapidly than the corresponding subunits in a tetramer. Hargrove et al. (19) have shown that the association rate constant for the binding of monomeric heme to apoglobins is always $~1 \times 10^8$ M⁻¹ s⁻¹ regardless of the exact protein structure. Thus, the equilibrium constant for hemin dissociation can be computed as $K_{H} = k_{-H} / (1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1})$ where $k_{-H}$ is converted from units of h⁻¹ to s⁻¹. Equilibrium dissoci-
for hemin may explain why it is difficult to express the precipitate almost immediately after heme removal. Aggregation and precipitation. In addition, the apoprotein phase (native) for why the aquomet forms of isolated subunits of human hemoglobin separately as soluble holoprotein is unstable. The half-times for hemin dissociation are 1–3 min for sperm whale myoglobin at pH 7, 37 °C.

Formation of α1β2 dimers from monomers causes a 30-fold increase in the affinity of α subunits for hemin, whereas only a 2-fold increase is observed for β subunits. The structural cause of this selectivity is not clear since the α1β2 interface involves mostly hydrophobic contacts between the B, G, and H helices of the two subunits, regions which are far removed from the heme pocket in both proteins. Presumably, formation of these complexes stabilizes the overall, tertiary structure of α but not β subunits. Association of dimers into tetramers is driven primarily by formation of the α1β2 interface which involves more polar contacts between the C and N termini and the C-helices and FG corners of both subunits. The C-helix is near the heme group and the FG corner serves to position the proximal His(F8) for direct coordination to the iron atom. These interactions are required for strong hemin binding to β subunits. A more detailed structural interpretation will require systematic mutagenesis studies analogous to those carried out for sperm whale myoglobin (24).

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TABLE II
Comparison of the equilibrium dissociation constants for hemin binding to the α and β subunits of native human hemoglobin and sperm whale myoglobin at pH 7.0, 37 °C

| Protein       | Kd monomer (pM) | Kd dimer (pM) | Kd tetramer (pM) |
|---------------|-----------------|---------------|------------------|
| Sperm whale myoglobin | 0.03 | 3.3 | 1.7 |
| α (native)    | 33              | 110           | 42               |
| β (native)    | 108             | 42            | 4.2              |

The K_d values in picomolar were calculated using the dissociation rate constants in Table I and assuming that the association rate constant is ~1 × 10^-10 M^-1 s^-1 for all three apoproteins, regardless of quaternary state (24).

FIG. 5. Hemin dissociation from isolated α and β subunits and intact native methemoglobin at pH 7, 37 °C. The concentrations of all three proteins was ~10 μM after mixing in a stopped flow apparatus as described in the text. The hemin dissociation rates for the β and α subunits in native hemoglobin were 7 and 0.5 h^-1, respectively. Hemin dissociation from isolated α chains showed a single phase with k_d = 12 h^-1. Hemin dissociation from isolated β chains showed a fast phase (k_d ~ 40 h^-1), presumably associated with β monomers, and a slower phase (k_d ~ 2 h^-1) associated with β tetramers.

TABLE II
Hemoglobin: Molecular, Genetic, and Clinical Aspects, Chap. 16, pp. 634–662, W. B. Saunders Co., Philadelphia

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