Aggregation of Deoxyhemoglobin Subunits*

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The formation of deoxyhemoglobin was examined by measuring the heme spectral change that accompanies the aggregation of isolated \( \alpha \) and \( \beta \) chains. At low heme concentrations (<10^{-5} \text{ M}), tetramer formation can be described by two consecutive, second order reactions representing the aggregation of monomers followed by the association of \( \alpha \beta \) dimers. At neutral pH, the rates of monomer and dimer aggregation are roughly the same, \( \approx 5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) at 20°C. Raising or lowering the pH results in a uniform decrease of both aggregation rates due presumably to repulsion of positively charged subunits at acid pH and repulsion of negatively charged subunits at alkaline pH. Addition of \( p \)-hydroxymercuribenzoate to \( \alpha \) chains lowers the rate of monomer aggregation whereas addition of mercurials to the \( \beta \) subunits appears to lower both the rate of monomer and the rate of dimer aggregation.

At high heme concentrations (>10^{-5} \text{ M}) or in the presence of organic phosphates, the rate of chain aggregation becomes limited, in part, by the slow dissociation of \( \beta \) chain tetramers. In the case of inositol hexaphosphate, the rate of hemoglobin formation exhibits a bell-shaped dependence on phosphate concentration. When intermediate concentrations of inositol hexaphosphate (>10^{-4} \text{ M}) are preincubated with \( \beta \) subunits, a slow first order time course is observed and exhibits a half-time of about 8 min. As more inositol hexaphosphate is added, the chain aggregation reaction begins to occur more rapidly. Eventually at about 10^{-2} \text{ M} inositol hexaphosphate, the time course becomes almost identical to that observed in the absence of phosphates. The increase in the velocity of the chain aggregation reaction at high phosphate concentrations suggests strongly that inositol hexaphosphate binds to \( \beta \) monomers and, if added in sufficiently large amounts, promotes \( \beta \), dissociation. A quantitative analysis of these results showed that the affinity of \( \beta \) monomers for inositol hexaphosphate is the same as that of \( \alpha \beta \) dimers. Only when tetramers are formed, either \( \alpha \beta \) or \( \beta \), is a marked increase in affinity for inositol hexaphosphate observed.

In 1965 Bucci and Fronticelli (1) reported a procedure for isolating the \( \alpha \) and \( \beta \) subunits of human hemoglobin in a functionally intact form (i.e. capable of reversible combinations with heme ligands). Subsequently, Antonini and co-workers (2, 3) showed that the separated chains react rapidly with CO and bind oxygen 30 to 40 times more tightly than hemoglobin. In the deoxygenated, ferro state, the subunits exhibit a modified Soret absorption spectrum (4, 5) which is analogous to that of the Hb* species first observed by Gibson (7) in flash photolysis experiments. The difference between the absorption properties of the isolated chains and those of intact deoxyhemoglobin was used by Antonini et al. (4) to monitor tetramer formation in conventional rapid mixing experiments. When equimolar solutions of unliganded \( \alpha \) and \( \beta \) chains were mixed in a stopped flow apparatus, the resultant absorbance change exhibited a marked lag phase (4). This was followed by a nonexponential time course which was indicative of a second order association reaction. The half-time of the overall reaction was proportional to the reciprocal of the initial total heme concentration. Antonini et al. (4) interpreted these results in terms of the following scheme:

\[
\begin{align*}
\alpha^* + \beta^* & \rightarrow \alpha \beta^* \quad \text{(a)} \\
\alpha \beta & \rightarrow \alpha \beta^* \quad \text{(b)} \\
\alpha \beta^* & \rightarrow \alpha \beta \quad \text{(c)} \\
2\alpha \beta & \rightarrow \alpha \beta_2 \quad \text{(d)}
\end{align*}
\]

where the starred species represent rapidly reacting, high affinity conformations. The majority of the absorbance change accompanying subunit aggregation was attributed to the isomerization of deoxyhemoglobin dimers (Equation 1c). This

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Abbreviations used are: Hb*, a rapidly reacting high affinity conformation of hemoglobin (an R state in terms of the allosteric, two-state model) which has been assigned to isolated chains, dimers, the last heme site to react with ligands (Hb,X), and certain high affinity mutant and chemically modified hemoglobins (6); inositol-\( P_6 \), inositol hexaphosphate; \( \text{hi}-\text{Tris} \), \( 2,3 \)'-\( \text{hi}(\text{hydroxymethyl}) \)-2,2',2''-nitrilotriethanol.
idea was based on Antonini's (8) belief at that time that deoxyhemoglobin dimers exhibit cooperative ligand binding. Since the sedimentation coefficient for isolated β chains was observed to be around 4 S (4) and that for α chains around 1.9 S, the β subunits were postulated to exist as tetramers until recombined with monomeric α chains. The dissociation of β chain oligomers (Equation 1a) was thought to account for the majority of the lag phase seen in the stopped flow traces. The second order nature of the remaining portion of the time course was assigned to the aggregation of monomeric units (Equation 1b).

Since the original studies of Antonini et al. (4), several workers (9-11) have shown that unliganded dimers exhibit Fe⁺⁺ spectral and functional characteristics and do not bind ligands in a cooperative manner. Consequently, most, if not all, of the subunit aggregation absorbance change is associated with tetramer formation, the last step in Equation 1. In addition, the contention of Antonini et al. (4) that βₔ dissociation accounts for the lag phase is inconsistent with the heme concentration dependence of their observed time courses. At micromolar concentrations, the half-time of the absorbance change was proportional to the reciprocal of the initial heme concentration. This relationship would not be observed if a first order dissociation reaction were limiting the overall aggregation rate. As Gibson (6) has suggested, the lag phase is more likely to be due to the lack of absorbance change associated with dimer formation.

In view of these more recent results and interpretations, we have attempted to describe the formation of deoxyhemoglobin at low heme concentrations, using only Equations 1b and 1d. Quantitative analysis of the absorbance time courses required the assignment of only a single rate constant since the rate of monomer aggregation, k₁, was allowed to vary. As shown in Fig. 1C, the halftime of the overall aggregation rate was obtained by removing the alkyl ammonium cation on Bio-Rad AG 50W-X8 cation exchange resin, concentrating the acid form of the glycerate by flash evaporation, and neutralizing the resultant solution with NaOH. Concentrations were determined by standard phosphate analysis. In all cases, small amounts of sodium dithionite (Eastman) were added to the anaerobic chain solutions to scavenge any residual O₂ bound to the isolated subunits. When p-hydroxymercuribenzoate was added to the chains, this was always done prior to dithionite addition.

**MATERIALS AND METHODS**

Human hemoglobin was prepared as described by Wiedemann and Olson (12). Isolated α and β chains were prepared by the method of Geraci et al. (13) with the following exception: p-mercuribenzoate was removed from the β chains by passage through a Sephadex G-25 column equilibrated with 0.1 M 2-mercaptoethanol (14). Regeneration of the sulphydryl groups by the DEAE-cellulose procedure of Geraci et al. (13) consistently yielded large populations of β chains (up to 50%) that were inactive with respect to hemoglobin formation. Although they exhibited normal heme spectra and reacted rapidly with heme ligands, a large portion of these β chain preparations were incapable of recombining with α chains, even after several hours of incubation. In contrast, the Sephadex column procedure of Sugita et al. (14) consistently yielded fully active β chain preparations as judged by electrophoresis and the magnitude of the absorbance change accompanying tetramer formation.

The isolated chains were judged to be free of mercury by titration (Fig. 1A) and to P-heme concentration when the β subunits were anaerobic. The Soret difference spectrum observed in these experiments agreed well with the chain aggregation difference spectrum reported by Brunori et al. (5) and with previously reported spectra for the association of deoxyhemoglobin dimers (9-11). We chose to follow subunit aggregation at the 445 nm difference minimum rather than at the 430 nm maximum, even though the change is roughly 2-fold larger. The lower background absorbance at 445 nm improved significantly the signal to noise ratio of the traces and reduced nonlinearity between absorbance changes and concentration due to deviations from Beer’s law (i.e. optical artifacts due to polychromatic light).

A series of time courses in which one or both of the chain concentrations was varied are shown in Fig. 1. The total absorbance change was directly proportional to the α-heme concentration when the β subunits were present in excess (Fig. 1A) and to β-heme concentration when the α subunits were in excess (Fig. 1B). These results show that both chain preparations are fully reactive with respect to hemoglobin formation and that there are no significant deviations from Beer’s law over the heme concentration range used (2 to 15 x 10⁻⁶ M). As shown in Fig. 1C, the half-time of the overall reaction increases 4-fold, from 1.4 to 5.6 s, in going from 8.9 to 2.0 x 10⁻⁶ M total heme concentration after mixing. This hemoglobin concentration dependence agrees well with that reported by Antonini et al. (4) and indicates little contribution from a first order αₔ dissociation rate.

The time courses in Fig. 1 were analyzed in terms of the following scheme:

**RESULTS**

The absorbance time courses shown in Fig. 1 were fitted to Equations 2. For each trace, the absorbance changes were normalized and then used to calculate tetramer concentrations. In all cases, the rate of dimer aggregation, k₂, was fixed at 5.2 x 10⁴ M⁻¹ s⁻¹ (12), whereas the rate of monomer aggregation, k₄, was allowed to vary. S represents the standard deviation of the fitted curve in absorbance units.

| Concentration of α before mixing | Concentration of β before mixing | k₄ x 10⁻⁴ M⁻¹ s⁻¹ | S |
|---------------------------------|---------------------------------|-------------------|---|
| 14.9                           | 15.0                            | 4.4               | ±0.005 |
| 7.5                            | 15.0                            | 3.5               | ±0.001 |
| 3.7                            | 15.0                            | 4.1               | ±0.003 |
| 1.9                            | 15.0                            | 9.8               | ±0.001 |
| 14.9                           | 15.0                            | 4.0               | ±0.005 |
| 14.9                           | 7.5                             | 3.5               | ±0.002 |
| 14.9                           | 3.7                             | 3.0               | ±0.002 |
| 14.9                           | 1.9                             | 2.3               | ±0.001 |
| 8.0                            | 8.0                             | 5.2               | ±0.001 |
| 2.0                            | 2.0                             | 5.0               | ±0.001 |
Formation of Deoxyhemoglobin

Fig. 1. The aggregation of isolated α and β chains in 0.1 M bis-Tris, 0.1 M NaCl at pH 7.0, 20°. The reactions were followed in the stopped flow apparatus at 445 nm through a 1.5-cm path length cell. The circles represent experimental points. The lines represent best fits of Equation 2 to the experimental data. Values of the rate constants used to calculate these lines are given in Table I. A, the reaction of 15.0 × 10^{-3} M β chains (before mixing) with varying α chain concentrations. B, the reaction of 14.9 × 10^{-3} M α chains (before mixing) with varying β chain concentrations. C, the reaction of equimolar concentrations of chains. ΔA represents the absorbance of the reaction mixture at any time minus that after the reaction is completed. Note that the ordinate is log (ΔA) and therefore, deviations near the end of the reactions tend to appear magnified out of proportion to their actual absorbance change differences.
All of the heme absorbance change was attributed to the dimer association step, Equation 2b. The rate of this reaction, $k_2$, was fixed at $5.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, the value reported by Wiedermann and Olson (12) for the rate of dimer aggregation in 0.1 M bis-Tris, 0.1 M NaCl at pH 7.0, 20°C.

As shown by the correspondence between the experimental points and the calculated lines in Fig. 1, Equation 2 provides a good quantitative description of the time course of tetramer formation. It is particularly satisfying that the dimer association rate measured independently by pH drop experiments (12) applies quantitatively to the overall subunit aggregation process. The average value of $k_1$, obtained from 24 separate fits using four different chain preparations was $5.0 \pm 1.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ for the reaction in 0.1 M bis-Tris, 0.1 M NaCl at pH 7.0, 20°C. An analysis of a number of experiments similar to those reported in Fig. 1 indicated that the optimum condition for determining the value of $k_1$ was to mix equimolar concentrations of $\alpha$ and $\beta$ chains at approximately $8 \times 10^{-5}$ M total heme. Under these conditions, the absorbance change is relatively large, 0.06 to 0.08, whereas deviations from Beer’s law and contributions from the dissociation of $\beta$ oligomers are still negligible (see "Discussion").

Effects of Protons and p-Hydroxymercuribenzoate—In the range pH 7 to 8, there is little dependence of the time course of chain aggregation on proton concentration (Fig. 2, Table II, and Ref. 4). However, at either low or high pH, there is a definite decrease in the velocity of the reaction which results from a relatively uniform lowering of both $k_1$ and $k_2$ (Table II). This decrease in rate is probably due to unfavorable electrostatic interactions between positively charged subunits at acid pH and the negatively charged subunits at alkaline pH.

In agreement with the results of Antonini et al. (4), the addition of p-hydroxymercuribenzoate to either one or both of the chains causes a marked decrease in the velocity of the aggregation reaction. The addition of 1 eq of mercurial to $\beta$ chains appears to cause a decrease in both the rate of monomer and the rate of dimer aggregation (Table II and Fig. 3A). In contrast, the addition of mercurial to $\alpha$104 cysteine appears to affect only the rate of dimer formation (Table II and Fig. 3B). These results are consistent with the currently accepted idea that the $\alpha_1\beta_1$ interface is formed during monomer association and the $\alpha_1\beta_2$ interface during dimer association (9-11, 22, 24).

Since $\alpha$104 cysteine is located at the $\alpha_1\beta_1$ interface, modification of this residue would be expected to affect the rate of monomer association, but a priori, there is no reason to expect an effect on the rate of dimer aggregation (i.e. $\alpha_1\beta_2$ interface formation). McDonald and Noble (25) have shown that cysteines 93 and 112 of isolated $\beta$ chains react at equivalent rates with p-hydroxymercuribenzoate. Therefore, when one p-hydroxymercuribenzoate per heme is added to $\beta$ chains, 50% of the subunits should be modified at $\beta$93 cysteine and 50% at $\beta$112 cysteine. Since $\beta$112 cysteine is located in the $\alpha_1\beta_1$ interface and $\beta$93 cysteine near the $\alpha_1\beta_2$ interface, 1 eq of mercurial added to the $\beta$ subunits would be expected and is observed to influence the rate of both steps in the overall chain aggregation process (Table II). The presence of mercurial at both $\alpha$104 cysteine and $\beta$112 cysteine decreases the rate of monomer aggregation even further, lowering the value of $k_1$ to about $0.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$.

Effects of Organic Phosphates—The effect of inositol-$P_6$ on
the chain aggregation process depends on whether or not the organic phosphate is preincubated with β subunits (Fig. 4A). When \(3 \times 10^{-6} \) M inositol-P<sub>6</sub> is added to β chains prior to mixing, the resultant time course is markedly altered. First, the total absorbance change decreases by about 30%. This suggests that the binding of inositol-P<sub>6</sub> perturbs the heme absorption spectrum of β chains. Second, the remaining absorbance change exhibits an extremely slow, first order time course with a half-time of about 8 min. In contrast, when inositol-P<sub>6</sub> is present only in the α chain solution, the velocity of the overall aggregation reaction increases 2-fold over that measured in the absence of phosphates.

These results are readily explained by assuming that inositol-P<sub>6</sub> perturbs the \(4\beta = \beta_4\) equilibrium and causes the formation of slowly dissociating tetramers containing bound phosphate. At the low heme concentrations given in Fig. 4A, preincubation of the β chains with inositol-P<sub>6</sub> is required for the observation of slowly dissociating tetramers since the aggregation of β monomers is a slow process (see Fig. 7). If the phosphate is present only in the α chain solution, \(\alpha\beta\) dimer formation competes effectively for β monomers, thereby inhibiting β chain polymerization. Under these conditions any slow phase that is observed represents the portion of β chains which are present initially as tetramers in the absence of phosphates. The small fraction of slow phase (≤5 to 10%) that is seen in Fig. 4A, when only the α chains are pretreated with inositol-P<sub>6</sub>, is further evidence that most of the β chains are monomeric at \(8 \times 10^{-6}\) M heme.

These interpretations are supported by results at higher heme concentrations (Fig. 4B). At \(80 \times 10^{-6}\) M heme, the reaction of α chains containing inositol-P<sub>6</sub> with untreated β chains exhibits a slow phase which comprises 70 to 80% of the total absorbance change. This indicates that the majority of the β chains are present as tetramers prior to mixing. The time course observed in the absence of phosphates also exhibits behavior indicative of a substantial population of β chain oligomers. The half-time of the overall reaction at \(80 \times 10^{-6}\) M heme is nearly equal to that observed at \(8 \times 10^{-6}\) M heme and approximately 2 times greater than that observed at

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**Fig. 3.** The effect of \(p\)-mercuribenzoate modification on the rate of chain aggregation in 0.1 M bis-Tris, 0.1 M NaCl at pH 7.0, 20°. Equimolar concentrations of α and β chains (\(8 \times 10^{-6}\) M total heme after mixing) were mixed and the reactions followed at 445 nm. \(\Delta A/\Delta A_0\) represents the normalized absorbance change (see Fig. 1). The solid lines represent best fits to Equation 2 and were calculated using the parameters listed in Table II. A, effect of mercurial substitution on the α chains.

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**Fig. 4.** The effect of organic phosphates on the aggregation of α and β chains in 0.1 M bis-Tris, 0.1 M NaCl at pH 7.0, 20°. In all cases, equimolar concentrations of α and β chains were mixed together in the stopped flow apparatus and the reactions followed through a 1.5-cm cuvette. The lines were drawn through the experimental points and do not represent theoretical fits. A, effect of inositol-P<sub>6</sub> (IP<sub>6</sub>) at \(8 \times 10^{-6}\) M total heme after mixing. Reactions were measured at 445 nm. O, untreated α chains plus untreated β chains; C, α chains containing \(10^{-4}\) M inositol-P<sub>6</sub> before mixing plus phosphate-free β chains; Δ, phosphate-free α chains plus β chains preincubated with \(3 \times 10^{-6}\) M inositol-P<sub>6</sub> before mixing. B, effect of inositol-P<sub>6</sub> (IP<sub>6</sub>) at \(80 \times 10^{-6}\) M total heme after mixing. Reactions were measured at 590 nm. O, untreated α chains plus untreated β chains; C, α chains containing \(10^{-4}\) M inositol-P<sub>6</sub> before mixing plus phosphate-free β chains; Δ, phosphate-free α chains plus β chains preincubated with \(3 \times 10^{-6}\) M inositol-P<sub>6</sub> before mixing. C, effect of 2,3-diphosphoglycerate (DPG) at \(1.5 \times 10^{-5}\) M total heme after mixing. The reactions were measured at 445 nm. O, untreated α chains plus untreated β chains; C, α chains containing \(1.2 \times 10^{-5}\) M 2,3-diphosphoglycerate before mixing plus phosphate-free β chains; Δ, α chains containing \(1.2 \times 10^{-6}\) M 2,3-diphosphoglycerate plus β chains preincubated with \(1.2 \times 10^{-6}\) M 2,3-diphosphoglycerate.
15 × 10^-5 m heme (Fig. 4C). This nonproportionality between half-time and reciprocal heme concentration implies a significant contribution from a slow, first order dissociation process.

As shown in Fig. 4C, 2,3-diphosphoglycerate exerts an effect on chain aggregation which is similar to that observed with inositol-P6. Again, when the β chains are preincubated with the organic phosphate, a slow first order phase is observed and contributes a majority of the absorbance change. In the case of 2,3-diphosphoglycerate, the half-time of the slow phase is 18 s. The major difference between 2,3-diphosphoglycerate and inositol-P6 is the inability of the diphosphate to increase significantly the velocity of the overall chain aggregation process when it is added to the α chains alone.

**Acceleration of Tetramer Formation**—At 8 × 10^-6 m heme when inositol-P6 is present in the α chain and not the β chain solution, the time course for chain aggregation exhibits a rate which is 2-fold greater than that seen in the absence of phosphates (Fig. 4A). This observation requires that inositol-P6 binds to intermediates in the reaction (i.e., monomers or αβ dimers) and increases their rate of aggregation. The velocity of the overall reaction reaches a maximum at about 10^-1 m inositol-P6 and then decreases back to the original rate at much higher phosphate concentrations (Fig. 5A). This "bell-shaped" dependence is analogous to that observed by Wiedermann and Olson (12) for the dependence of the rate of dimer association on inositol-P6 concentration. These authors showed that the rate of association of a dimer containing bound phosphate with a phosphate-free dimer is much greater than either the rate of association of two phosphate-free dimers or the rate of association of two dimers, both containing bound phosphate.

The resultant dimer aggregation rate, k, in Equation 2, is given by:

\[
k = \frac{k_{\text{b}}(K + k_{\text{D}}(X) + k_{\text{D}}(X))^2}{\alpha + (X)^2}
\]

where K is the equilibrium dissociation constant for the binding of inositol-P6 to αβ dimers; k_{\text{b}}, the rate of association of phosphate free dimers; k_{\text{D}}(X), the rate of association of unlike dimers, one with phosphate bound and one without; k_{\text{D}}(X), the rate of association of phosphate containing dimers; and X represents inositol-P6.

A series of α (inositol-P6) + β time courses were measured in which the inositol-P6 concentration was varied from 0 to 5 × 10^-5 m, and the results were analyzed in terms of Equation 2. Only the value of k_{\text{b}} was allowed to vary; k_{\text{b}} was fixed at values calculated from Equation 3 using the parameters measured by Wiedermann and Olson (12) in 0.1 M bis-Tris, 0.1 M NaCl at pH 7.0, 20°. Equimolar concentrations (8 × 10^-6 m total heme after mixing) of α and β chains were mixed and the reactions followed at 445 nm. For these experiments inositol-P6 (IP6) was added to the α chain solutions only to prevent the formation of β chain tetramers. As mentioned in the text a slow first order phase (t_{1/2} ~ 10 to 20 min) is observed in the α (inositol-P6) + β experiments but comprises only 5 to 10% of the total absorbance change. This slow phase was subtracted from the total changes, and the resultant fast phase was analyzed in terms of Equation 2. A, normalized time courses for α (inositol-P6) + β. The concentration of inositol-P6 after mixing is shown beside each curve. The solid lines represent fits to Equation 2. k_{\text{b}} was fixed as described in the text and k_{\alpha} allowed to vary. Values of k_{\text{b}} and k_{\alpha}, respectively, were: 5.0 × 10^-5 s^-1, 5.2 × 10^-5 s^-1 for no inositol-P6; 11.0 × 10^-5 s^-1, 27.4 × 10^-5 s^-1 for 7.5 × 10^-5 m inositol-P6; 5.9 × 10^-5 s^-1, 10.6 × 10^-5 s^-1 for 1 × 10^-5 m inositol-P6. B, dependence of the rates of monomer, k_{\text{m}}, and dimer, k_{\text{d}}, aggregation on inositol-P6 concentrations. Values of k_{\text{m}} were calculated from Equation 3 as described in the text. Values for k_{\text{d}} were obtained from fits to Equation 2. The concentration of inositol-P6 is given as that after mixing.

β monomers. Consequently, at very high concentrations inositol-P6 causes the dissociation of β tetramers. When this dissociation is substantial, the chain aggregation reaction exhibits a "normal," rapid time course (the 50,000 μm curve in Fig. 6).

When the β chains are preincubated with 3 × 10^-5 m inositol-P6, the total aggregation absorbance change decreases roughly 30% (the 30 μm curve in Fig. 6). However, as the inositol-P6 concentration is raised further, the aggregation absorbance change begins to increase and eventually approaches and surpasses slightly the magnitude of the changes observed in the absence of phosphates. A direct demonstration of the effect of inositol-P6 on the β-heme spectrum is shown in Fig. 7. A large difference spectrum is observed when β chains are mixed with 0.5 to 1.5 × 10^-4 m inositol-P6. However, little absorbance change is seen when the experiment is repeated using a thousand-fold higher phosphate concentration. The β chain difference spectrum at intermediate phosphate concentrations is different from the overall difference spectrum produced by mixing unliganded α and β chains. The β chain spectrum is smaller in magnitude and appears to represent a
blue shift of the Soret absorption peak. As shown in the inset of Fig. 7, the reaction of β chains with inositol-P₆ can be followed in the stopped flow apparatus. At 8 x 10⁻⁶ M heme almost all of the absorbance change occurs slowly and exhibits a higher order kinetic pattern indicative of monomer aggregation.

The following equilibria,

\[
\begin{align*}
K_1 &= \frac{[\beta\beta]}{[\beta\gamma]} \quad \text{(4)} \\
K_2 &= \frac{[\beta\gamma]}{[\beta\beta]} \quad \text{(5a)} \\
K_3 &= \frac{[\beta]}{[\beta\gamma]} \quad \text{(5b)}
\end{align*}
\]

where X represents inositol-P₆, were used to analyze the results in Figs. 6 to 8. This scheme assumes that β dimers are thermodynamically unstable; that is, \(K_1\) is the product of a very small number representing dimer formation and a very large number representing tetramer formation from dimers (see "Discussion" and Footnote 3). The fraction of slowly dissociating (with a rate \(=10^{-8} \text{ s}^{-1}\)) or spectrally altered β chains is given by:

\[
\gamma = \frac{\frac{4}{3} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)^2}{[C_0]} - \frac{\frac{3}{2} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)}{1 + \frac{[\beta\gamma]}{[\beta\beta]}} (1 - f_\beta) \quad \text{(5c)}
\]

where \(C_0\) is the total heme concentration; \(\langle X \rangle\), the concentration of unbound inositol-P₆ and \(f_\beta\), the fraction of β monomers. The fraction of β monomers is given by the appropriate root (0 < \(f_\beta < 1\)) of the following polynomial:

\[
f_\beta = \left(\frac{4}{3} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)^2 \frac{[\beta]}{[\beta\gamma]} \frac{[\beta\gamma]}{[\beta\beta]} \left(1 + \frac{[\beta\gamma]}{[\beta\beta]} \frac{[\beta]}{[\beta\gamma]} \right)^{-1} - \frac{\frac{3}{2} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)}{1 + \frac{[\beta\gamma]}{[\beta\beta]}} (1 - f_\beta) \right)^{-1}\]

\[
[\beta\gamma] = \left(\frac{\frac{4}{3} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)^2}{[C_0]} - \frac{\frac{3}{2} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)}{1 + \frac{[\beta\gamma]}{[\beta\beta]}} (1 - f_\beta) \right)^{-1} \quad \text{(5a)}
\]

The fraction of slowly dissociating (with a rate \(=10^{-8} \text{ s}^{-1}\)) or spectrally altered β chains is given by:

\[
\gamma = \frac{\frac{4}{3} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)^2}{[C_0]} - \frac{\frac{3}{2} \left(\frac{[\beta\gamma]}{[\beta\beta]}\right)}{1 + \frac{[\beta\gamma]}{[\beta\beta]}} (1 - f_\beta) \quad \text{(5c)}
\]

A summary of β chain polymerization data is shown in Fig.
where the fractional $\beta$-heme spectral change or the fraction of slowly dissociating $\beta$ tetramers is plotted versus the logarithm of the total inositol-P$_4$ concentration. The solid line represents a best fit to the experimental data and was calculated from Equation 5 using the following parameters: $K_1 = 8 \times 10^{-6}$ M$^{-2}$, $K_2 = 10^4$ M$^{-1}$, $K_3 = 10^6$ M$^{-1}$, and $C_0 = 8 \times 10^{-6}$ M. The fitted value of $K_1$ predicts 10% tetramers on a per heme basis at $8 \times 10^{-6}$ M $\beta$ chains and 74% tetramers at $8 \times 10^{-5}$ M. These percentages of $\beta$ tetramers agree well with those calculated from the $\alpha$(inositol-P$_4$) + $\beta$ experiments shown in Fig. 4, A and B.

The results in Fig. 8 indicate that, at high concentrations, inositol-P$_4$ binds to $\beta$ monomers. In contrast to binding to tetramers, binding to monomers appears to have little effect on the spectral properties of the $\beta$ heme groups (open circles, Fig. 7). Similarly, bound inositol-P$_4$ does not appear to influence the reactivity of $\beta$ monomers with $\alpha$ chains. As shown in Fig. 5B, the rate of $\alpha$ dimer formation does not vary significantly with inositol-P$_4$ concentration, even in the range where binding to $\beta$ monomers takes place. This contrasts with the marked changes in the rate of tetramer formation as inositol-P$_4$ is bound to $\beta$ dimers ($k_2$ in Fig. 5B).

**Discussion**

In the range $10^{-4}$ to $10^{-2}$ M heme, the aggregation of deoxygenated hemoglobin subunits can be described by two consecutive, second order reactions: association of $\alpha$ and $\beta$ monomers followed by the association of $\alpha$-$\beta$ dimers (Equation 2). These reactions are effectively irreversible at pH 7.0. In the absence of mercurials and denaturing agents, no evidence has been reported for the presence of monomers in hemoglobin solutions. Similarly, the equilibrium constant for tetramer to dimer dissociation of deoxyhemoglobin is of the order of $10^{-18}$ to $10^{-12}$ M at neutral pH (26, 27). At pH 7.0, 20%, the rates of monomer and dimer aggregation are approximately the same, $5 \times 10^{-1}$ M$^{-1}$ s$^{-1}$. Thus, the rate of formation of the thermodynamically more stable $\alpha\beta\beta_2$ interface is essentially identical to the rate of formation of the less stable $\alpha\beta_2$ interface. Consequently, the kinetic expression of the difference in stabilities of the two sets of intersubunit bonds must reside in the dissociation rate constants. The velocity of tetramer to dimer dissociation must be greater than the velocity of dimer to monomer dissociation.

At higher heme concentrations, evidence is observed for the presence of $\beta$ chain oligomers. For example, the half-times of the chain aggregation reactions at $8 \times 10^{-6}$ and $80 \times 10^{-6}$ M heme are the same but the characteristics of the time courses are markedly different (Fig. 4). The absorbance trace observed at $8 \times 10^{-6}$ M is readily analyzed in terms of Equation 2 and exhibits a marked lag phase representing the association of $\alpha$ and $\beta$ monomers. The time course at $80 \times 10^{-6}$ M cannot be fit to Equation 2. Although at $80 \times 10^{-6}$ M heme the initial portion of the absorbance change does occur more rapidly, the latter portion is considerably slower than that at $8 \times 10^{-6}$ M heme. The net result is a markedly biphasic time course at the higher protein concentration.

We have attempted to analyze the time course at $80 \times 10^{-6}$ M heme by adding a $\alpha_4 \rightarrow 4\beta$ dissociation reaction to Equation 2 and dividing the initial $\beta$ chain population into monomeric and tetrameric fractions. The best fit was obtained by setting the $\beta$ dissociation rate equal to $0.25$ s$^{-1}$, assigning 70% of $\beta$ chains as tetramers initially, and fixing the values of $k_1$ and $k_2$ at 5.0 and $5.2 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively. The fraction of $\beta$ tetramers obtained from this fit agrees well with that estimated from the reaction of $\alpha$ chains containing inositol-P$_4$ with untreated $\beta$ chains (open circles, Fig. 4B). For the latter reaction ($\alpha$(inositol-P$_4$) + $\beta$), about 80% of the absorbance change occurred in a slow, first order phase representing the dissociation of $\beta$, containing bound phosphate. Since inositol-P$_4$ was not preincubated with the $\beta$ subunits the proportion of this slow phase represents the fraction of tetrameric $\beta$ chains present initially in the absence of phosphate.

Evidence for tetramers is also observed when $\beta$ chains are preincubated with organic phosphates, even at low heme concentration. Under these conditions, the rate of $\beta$ dissociation is extremely small, going from about $0.25$ s$^{-1}$ in the absence of phosphates to about $0.05$ s$^{-1}$ in the presence of $1.4$ mM 2,3-diphosphoglycerate and $0.0014$ s$^{-1}$ in the presence of $3 \times 10^{-4}$ M inositol-P$_4$. In the case of inositol-P$_4$, as the phosphate concentration is raised, binding to $\beta$ monomers occurs and eventually promotes complete dissociation of all $\beta$ molecules (Equation 5 and Fig. 8).

Quantitative analysis of the data in Fig. 8 indicated a value of $8 \times 10^{-3}$ M$^{-2}$ for the $4\beta \rightarrow \beta$ equilibrium constant. This value predicts negligible amounts of $\beta$ in the $10^{-6}$ M concentration range used in the stopped flow experiments shown in Figs. 1 to 3. However, the same value predicts the 74% of the $\beta$ chains should be tetramers at $8 \times 10^{-6}$ M. This agrees well with the percentage estimated from analyses of the observed chain aggregation time courses at $8 \times 10^{-3}$ M heme (Fig. 4B). The relatively sharp rise in the fraction of $\beta$, in going from $8 \times 10^{-6}$ to $8 \times 10^{-5}$ M heme reflects the fourth order nature of the $\beta$ chain aggregation reaction (Equation 5). In the past, most workers have assumed that $\beta$ chains are tetramers at all heme concentrations (4, 18, 28). This idea was based on sedimentation velocity studies which indicated 4 to 4.5 s$_{20,w}$ values for isolated $\beta$ chains. However, all of these studies used schlieren optics and, therefore, heme concentrations $\approx 10^{-4}$ M. Consequently, these high s$_{20,w}$ values do not conflict with our observations. In fact, the value of $K$, obtained from the data in Fig. 8 predicts s$_{20,w}$ values greater than 4.0 S at $10^{-1}$ M heme.

It is unrealistic to assume that $\beta$ tetramer formation is a concerted process requiring the simultaneous collision of 4 subunits. Similarly, the dissociation process probably occurs in two steps: $\beta_4 \rightarrow \beta_3 \rightarrow 4\beta$. The fit of the data in Fig. 4B to a scheme using Equation 2 and a $\beta_4 \rightarrow 4\beta$ step exhibited a systematic pattern of deviations between observed and calculated points. We interpret this to mean that at least two steps are involved in the dissociation of $\beta$ chain polymers. Similar evidence for $\beta_3$ intermediates is shown in the inset of Fig. 7. The time course observed when $\beta$ chains are reacted with inositol-P$_4$ cannot be analyzed in terms of a concerted $4\beta \rightarrow \beta_4$ reaction (i.e. a plot of the reciprocal of the absorbance cubed versus time is not linear). On the other hand, the limited data available on the molecular weight of deoxygenated $\beta$ chains as a function of heme concentration precludes any serious attempt at assigning individual rate and equilibrium constants to the association and dissociation of $\beta$ dimers. Furthermore, as shown in Fig. 8, Equation 4 does serve as a reasonable quantitative description of the effect of inositol-P$_4$ on the state of aggregation of $\beta$ subunits. This suggests that $\beta$ dimers are probably thermodynamically unstable.

Tainsky and Edelstein (29) have reported a limited sedimentation equilibrium study of the dissociation of the carbon monoxide form of isolated $\beta$ chains. Absorption optics were used so that micromolar heme concentrations could be examined. A weight average molecular weight of 43,000 was observed for $8 \times 10^{-6}$ M $\beta$-CO in 0.1 M phosphate at pH 7.6, 20°. The protein concentration dependence of the observed molecular weight was analyzed in terms of a tetramer to dimer dissociation, and an equilibrium constant of $10^{-4}$ M was
As shown in Figs. 6 to 8, the binding of inositol-P, to β tetramers causes a perturbation of their absorption spectrum. In contrast, binding to monomers at higher phosphate concentrations produces little change in the spectral properties of the heme groups. Bonaventura et al. (30) have shown that the binding of inositol-P, to β chains causes a 2- to 3-fold decrease in oxygen affinity and a 2-fold decrease in the rate of CO binding. These effects were not observed with 2,3-diphosphoglycerate and appear to reflect an oxygen linked conformational change induced by inositol-P, binding. The difference spectrum shown in Fig. 7 appears to represent the effect of this conformational change on the electronic properties of the p heme groups. However, even in the absence of inositol-P, the p-heme absorbance change produced by phosphate binding to β is reversed by the formation of αβ dimers. Therefore, hemoglobin formation results in a net perturbation of the spectral properties of both the α and β heme groups. However, even in the presence of inositol-P, the α heme absorbance change is greater than the β-heme change (Fig. 7 and Fig. 5 of Ref. 32). The ability of bound inositol-P, to enhance the reactivity of an αβ dimer with another phosphate-free dimer has been discussed by Wiedermann and Olson (17). It appears that the phosphate binds to the same positively charged, H helical region of the β subunits which is occupied in tetrameric hemoglobin (33). Once bound, inositol-P, produces a negatively charged surface which facilitates interaction with the corresponding negatively charged, β chain region of phosphate-free dimers. On the other hand, the α chain, H helical region is not involved in the αβ interface. Consequently, bound inositol-P, is neither expected nor observed to influence significantly the rate of monomer aggregation (Fig. 5B).

Wiedermann and Olson (12) reported that αβ dimers bind inositol-P, with an affinity constant equal to 0.5 x 10⁹ M⁻¹. More recently, Hensley et al. (34) have reported ultracentrifugation studies which predict an affinity constant equal to 1 x 10⁹ M⁻¹ for inositol-P, binding to methemoglobin dimers. These values are very similar to the affinity constant for inositol-P, binding to β monomers which was used to fit the data in Fig. 8 (K₄ = 1 x 10⁹ M⁻¹. Equations 4 and 5). Thus, the affinity of β chains for inositol-P, is not influenced by the monomer to dimer transition. However, when tetramers are formed, either αββ or ββ, a marked increase in affinity for inositol-P, is observed. This increase in affinity appears to be a reflection of the juxtaposition of the positively charged, H helical regions of the β subunits within the tetramers (33). In fact, the free energies of binding to these β chain domains are nearly additive. The free energy for binding to αβ dimers or to β monomers is roughly -5.4 kcal/mol whereas the free energy for binding to αββ or to ββ is between -10.7 to -12.1 kcal/mol (Refs. 33-35 and Fig. 8). In contrast, liganded tetrameric hemoglobin exhibits an intermediate free energy for the binding of inositol-P, = -8.0 kcal/mol (35, 36). This smaller value for liganded hemoglobin presumably reflects the partial shielding of the β chain, H helical regions which accompanies the low to high affinity, quaternary conformational change.

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