Communication

Acceleration of Tetramer Formation by the Binding of Inositol Hexaphosphate to Hemoglobin Dimers

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SUMMARY

The aggregation of deoxyhemoglobin dimers was studied by dropping the pH of a dilute solution of deoxyhemoglobin originally at high pH. In the presence of inositol hexaphosphate, a sharp increase in the rate of dimer association was observed. At higher concentrations of the phosphate, the rate decreased to a value close to that seen in the absence of phosphate. These observations require that inositol hexaphosphate binds to deoxyhemoglobin dimers. The dependence of the aggregation rate on phosphate concentration occurs because the reaction of a dimer containing bound phosphate with a phosphate-free dimer is 30 to 50 times faster than either the association of phosphate-free dimers or the association of dimers both containing bound phosphate.

Recently Gray (1) has reported evidence which suggests that the rate of aggregation of deoxyhemoglobin dimers is influenced by the presence of organic phosphates. In the presence of 1 mM 2,3-diphosphoglycerate he observed a 3-fold increase in the rate of the slow drift phase which accompanies the rapid removal of oxygen from solutions of dilute oxyhemoglobin. We have observed a similar increase in the rate of aggregation of deoxyhemoglobin dimers in the presence of inositol hexaphosphate using the pH drop method reported by Andersen et al. (2). For these experiments, deoxyhemoglobin in a weakly buffered solution at a high enough pH to promote dissociation is rapidly mixed with a strongly buffered solution at lower pH. The only absorbance change observed is that due to the association of deoxyhemoglobin dimers. The enhancement of the rate of aggregation in the presence of inositol-P61 requires that the phosphate molecule binds to hemoglobin dimers. Further experiments were carried out in order to quantitate this effect in terms of the equilibrium and kinetic constants needed to describe tetramer formation.

Human blood was obtained from a local hospital. The red cells were washed three times with 0.9% NaCl and lysed with an equal volume of glass-distilled water. The hemolysate was made up to 3% NaCl (w/v), centrifuged to remove cell debris, and dialyzed overnight against 0.03 M Na2HPO4 at 4°. Phosphate-free hemoglobin was prepared by passing a sample of dialyzed hemolysate through a Sephadex G-25 fine column (5 x 40 cm) which was equilibrated and eluted with 0.01 M Tris-HCl, pH 8.0 buffer containing 0.1 M NaCl. Fresh samples were prepared every 5 to 10 days. Deoxyhemoglobin was prepared in tonometers by alternately evacuating and flushing with purified N2. Heme concentrations were determined on either a Cary 118 C or a Varian spectrophotometer. Buffer solutions were deoxygenated by bubbling with N2, and sodium dithionite (Eastman) at concentrations of approximately 20 μM in the hemoglobin solutions and 60 μM in the buffer solutions was utilized to scavenge any residual oxygen. pH determinations were made on a Radiometer model 64 pH meter, and a Gibson-Durrum stopped flow apparatus was used for measuring the rates of the aggregation reactions. Observations were made at 440 nm through a 2-cm path length cell. Inositol-P61 was purchased from Sigma. The pH drop experiments were performed by mixing a 26 μM solution of deoxyhemoglobin in 0.01 M 2-aminocaproate-NaOH/0.1 M NaCl, pH 10.6 with either 0.2 μM bis-Tris-HCl/0.1 M NaCl, pH 6.0 or 7.0, or 0.2 μM Tris HCl/0.1 M NaCl, pH 8.0 in the stopped flow apparatus. Inositol-P61 was added to the buffer solutions when applicable.

In agreement with the work of Andersen et al. (2), initial pH drop experiments showed a reaction which exhibited second order behavior, indicative of tetramer formation. The Soret difference spectrum observed in these experiments agreed well both with that reported by Andersen et al. (2) for similar experiments and with that reported by Brunori et al. (3) for the association of isolated α and β chains. As expected, the noval extinction change at 440 nm decreased as the heme concentration was raised and markedly increased when 2 eq (1 per β-93 cysteine) of p-hydroxymercuribenzoate were added to the deoxyhemoglobin solution. The latter effect is due to the greatly enhanced dissociation of hemoglobin when the β-93 sulfhydryl groups are reacted with mercurials. At pH 10.6, deoxyhemoglobin reacted with p-MB appears to be completely dissociated into dimers. The observed extinction of 5700 M−1 cm−1 at 440 nm in the presence of p-MB is nearly identical with that observed for the aggregation of isolated chains and was used to calculate the observed second order rates of dimer aggregation and to estimate the fraction of heme present initially as dimers in untreated hemoglobin solutions at high pH.

No change in the rate of aggregation of deoxy dimers was seen when the final pH was varied from 6.0 to 8.0 or when the salt concentration in the buffer solution ranged from 0.05 to 2.0 M. However, when 1 mM inositol-P61 was included in the buffer solution, a 6-fold increase in the second order rate occurred. On further addition of inositol-P61, the apparent second order rate decreased, eventually approaching a value similar to that seen in the absence of phosphates (Fig. 1). As shown in the inset to Fig. 1, inositol-P61 affects only the apparent rate constant and exerts little or no influence either on the magnitude of the absorbance change or on the second order behavior of the aggregation reaction.

In order to account for the "bell-shaped" dependence of the second order aggregation rate on inositol-P61 concentration, the following set of reactions was proposed:

\[
\begin{align*}
\text{D} + \text{D} & \rightarrow \text{D}_2 \\
\text{D}_2 + \text{IP}_6 & \rightarrow \text{D}_2 \cdot \text{IP}_6 \\
\text{D}_2 \cdot \text{IP}_6 & \rightarrow \text{D}_2 + \text{IP}_6
\end{align*}
\]

\[
k_1
\]

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The abbreviations used are: inositol-P61, inositol hexaphosphate; p-MB, p-hydroxymercuribenzoate; bis-Tris, 2,2'bis(hydroxymethyl)-2,2',2"-nitriloethanol; p-MB hemoglobin, deoxyhemoglobin containing 2 eq of p-hydroxymercuribenzoate per tetramer; kobs, the observed second order rate constant for dimer association.
where $D$ represents deoxyhemoglobin dimers; $D_n$ tetramers; and $X$, inositol-1,4,5-P$3$. The first reaction represents the normal association of dimers in the absence of inositol-P$6$. The second reaction occurs when 1 dimer molecule containing bound inositol-P$6$ associates with a free dimer to form a tetrameric inositol-P$6$ deoxyhemoglobin. The third reaction describes the aggregation of dimers, both of which contain bound phosphate, and requires the release of 1 molecule of inositol-PC in order to form a tetrameric inositol-PC. Gray and Gibson (4) have shown that the binding of inositol-P$6$ to all forms of hemoglobin is too fast to be measured by rapid mixing techniques. Thus, the equilibrium distribution of phosphate among the dimeric and tetrameric species present initially is achieved within the dead time of the stopped flow apparatus. Since this phosphate equilibration process is much more rapid than the rate of aggregation of the dimers, the ratio of unbound to bound dimers is defined throughout the reaction by the free concentration of inositol-PC and the equilibrium dissociation constant for the binding of inositol-P$6$ to dimers, $K_D = (X)(D)/(DX)$. All three of the aggregation reactions can be considered irreversible, since the tetramer to dimer equilibrium dissociation constant for deoxyhemoglobin is on the order of $10^{-12}$ M (5), and inositol-P$6$ decreases this number to an even smaller value. Finally, because the magnitude of the spectral change produced by the aggregation of deoxyhemoglobin dimers is unaffected by the presence or absence of inositol-P$6$, it appears that an identical extinction change occurs in each of the three reactions. Therefore, the rate of the absorbance change at 440 nm is directly proportional to the rate of decrease of the total dimer population, $(D_t) = (D) + (DX)$. The exact relationship is given by the following rate law:

$$\frac{d(D_t)}{dt} = \left(\frac{k_1[D] + k_2[D](X) + k_3(X)}{(D_t)^2}\right) \left(D_t\right)^2$$

where the expression within the brackets describes the apparent second order rate constant.

As shown in Fig. 2, the dependence of the observed second order rate of dimer aggregation on inositol-P$6$ concentration is quantitatively described by Equation 4. The values for the second order rate constants ($k_1$, $k_2$, and $k_3$) and the inositol-P$6$ equilibrium dissociation constants for binding to dimers ($K_D$) are given in Table I. The enhancement of the observed second order dimer association rate at pH 6.0 and 7.0 is due to the reaction of an unbound dimer with a dimer containing bound inositol-P$6$ (Equation 2). A 30-fold increase in rate at pH 7.0 is observed in going from $k_1$ to $k_3$, whereas the value of $k_1$ is nearly equal to that observed for $k_1$ (Table I). Presumably a similar increase in rate due to 2,3-diphosphoglycerate binding would account for the effect seen by Gray. As shown in Fig. 2, the maximum rate enhancement occurs at an inositol-P$6$ concentration nearly equal to the equilibrium dissociation constant for binding to dimers ($K_D = 3.0 \times 10^{-4}$ M at pH 7.0, 20°). These results show clearly that inositol-P$6$ binds to dimers and that concentrations of phosphate required to observe the effects of this binding are not exceedingly high.

The major effect of decreasing the pH of the strongly buffered solution to pH 6.0 is a 50% increase in $k_3$, the rate of association of bound and unbound dimers. The affinity of the dimers is only slightly increased. Raising the pH to 8.0 inhibits markedly the binding of inositol-P$6$ to dimers, and, even when bound, the
effect on the aggregation rates is significantly decreased (at most a 4-fold increase of $k_z$ over $k_{ij}$) from that observed at pH 6.0 or 7.0.

Finally, the effect of bound p-hydroxymercurobenzoate on the various association rate constants is somewhat similar to that observed at pH 8.0. However, there appears to be little or no effect on the affinity of the dimers for inositol-P$_6$, since the $K_D$ for p-MB dimers is in the same range as that for untreated dimers. As shown in Table 1, the major effect of p-MB is to decrease by 10-fold $k_b$, the rate of aggregation of dimers both containing bound phosphate. This apparent ability of p-MB to prevent the full expression of the effects of bound inositol-P$_4$ has been observed in studies of other properties of hemoglobin and is discussed in more detail by Olson (6).

The observation of inositol-P$_4$ binding to hemoglobin dimers does not conflict with the well documented stoichiometry of 1 organic phosphate molecule bound per 4 hemes. Binding to dimers is only noticed when examining the kinetics of the aggregation process. At equilibrium, the population of dimers containing bound inositol-P$_4$ is vanishingly small when compared to the amounts of free dimers, free tetramers, and inositol-P$_4$ bound tetramers. This is summarized by the following scheme using the same notation as in Equations 1 to 3:

$$
\begin{align*}
D_2 + X & \rightleftharpoons D_2X \\
& \rightleftharpoons D_2X \\
& \rightleftharpoons D_2X
\end{align*}
$$

In the case of deoxyhemoglobin, values for $K_{4,2}$ and $K_T$ have been estimated to be $\sim 10^{-12}$ M and $\sim 10^{-8}$ M, respectively, at pH 7.0, 20° (4, 5). Using $3 \times 10^{-4}$ M for $K_D$ (Table I), a value for $K_{4,2}$ (i.e. $K_{4,2} = K_T/K_D^2$) was estimated to be $10^{-18}$ M, which in turn must be multiplied by the free concentration of inositol-P$_4$ to obtain the fractional amounts of deoxyhemoglobin dimers and tetramers present. Thus, only for liganded forms of hemoglobin can there be a significant effect of excess inositol-P$_4$ on the aggregation equilibria shown above. For example, in the case of methemoglobin at pH 7.0, $K_{4,2}$ is about $10^{-3}$ M (7), $K_T$ is approximately $3 \times 10^{-8}$ M (6), and assuming that liganded and unliganded dimers are conformationally equivalent (2) and therefore bind inositol-P$_4$ to the same extent, $K_D$ is still $3 \times 10^{-4}$ M. These numbers predict a value for $K'_{4,2}$ of about $3 \times 10^{-4}$ M so that the effective methemoglobin tetramer to dimer dissociation constant in the presence of 1 mM inositol-P$_4$ would be roughly $10^{-7}$ M, which is in agreement with the range of values reported by Hensley et al. (7).

Both the ability of inositol-P$_4$ to bind to dimers and its enhancement of the rate of the asymmetric aggregation reaction ($D + DX \rightarrow D_2X$, Equation 2) are readily interpreted in terms of the structural models presented by Perutz (8). Although it is clear that the integrity of the central cavity between the $\beta$ chains in the tetramer is not an absolute requirement for organic phosphate binding, it is very likely that the phosphate binding region on the dimers is also the positively charged $H$ helical region of the $\beta$ chains. Inositol-P$_4$ binding converts this positively charged $\beta$ chain surface into a negatively charged region, thereby facilitating its interaction with $H$ helical regions of phosphate-free dimers. The presence of bound inositol-P$_4$ on both dimers would simply reverse the sign of the unfavorable interactions found originally for phosphate-free molecules and therefore not be expected to enhance the rate of aggregation.

REFERENCES

Acceleration of tetramer formation by the binding of inositol hexaphosphate to hemoglobin dimers.
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