The Functional Properties of Hemoglobin Bethesda

\( (\alpha_2\beta_2^{145\text{His}}) \)

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SUMMARY

Hemoglobin Bethesda is a \( \beta \) chain variant of human hemoglobin in which histidine is substituted for tyrosine at position 145. From chemical and crystallographic studies, it has been postulated that the tyrosine normally found at this position is critical for the maintenance of a normal deoxy structure. Kinetic studies of the carbon monoxide and \( n \)-butyl isocyanide binding reactions show that deoxyhemoglobin Bethesda in low ionic strength buffers at \( \text{pH} \) 7.0 exhibits noncooperative, chain-like behavior. Under the same conditions, the Soret absorption spectrum of deoxyhemoglobin Bethesda is more like that of the isolated \( \alpha \) and \( \beta \) chains than that of the normal deoxyhemoglobin tetramer. In addition, reversible dissociation of deoxyhemoglobin Bethesda can be demonstrated by ultracentrifugation experiments. All of these results suggest that at neutral \( \text{pH} \) deoxyhemoglobin Bethesda exists in a conformation analogous to that postulated for liganded hemoglobin A. However, in the presence of inositol hexaphosphate, more normal, cooperative kinetic behavior is observed, subunit dissociation is reduced, and the absorption spectrum in the Soret region is found to be virtually identical with that of deoxyhemoglobin A.

Thus, the substitution of histidine for tyrosine 145 \( \beta \) drastically alters the ability of hemoglobin to form a normal deoxy structure. Studies of the dissociation of oxygen and \( n \)-butyl isocyanide from saturated hemoglobin Bethesda indicate, however, that this mutation does not alter the intrinsic reactivities of the \( \alpha \) and \( \beta \) heme sites within the liganded conformation. Rather, this amino acid substitution appears to inhibit the quaternary change to the deoxy conformation in such a manner that only on the addition of organic phosphates is behavior observed which resembles that of normal deoxyhemoglobin A.

The elucidation of the functional effects of several of these modifications has allowed Perutz and co-workers to designate those regions of hemoglobin structure which are mandatory for normal cooperative behavior (1–4). Based on these results and on detailed information about the conformational differences between oxy and deoxyhemoglobin, Perutz (1) has proposed a specific stereochemical mechanism of ligand binding which includes an explanation of the alkaline Bohr effect, of the effect of organic phosphates, and a structural interpretation of abnormal hemoglobin behavior.

In particular, this model requires that the structural integrity of the COOH-terminal residues of all four subunits be maintained for normal function. In deoxyhemoglobin these residues are fixed in rigid conformations which are stabilized by both inter- and intrasubunit salt linkages and by van der Waals and hydrogen bonding forces which hold the penultimate tyrosines of each chain in pockets between the F and H helices. These structures are presumed to help stabilize deoxyhemoglobin A in a low affinity form. Upon ligand binding, the tyrosines are ejected and the salt linkages broken, which results in the disruption of the tertiary and secondary structure of the COOH-terminal residues. When all of these inter- and intramolecular constraints are broken, the hemoglobin molecule is thought to relax to its liganded or high affinity form. In hemoglobin Bethesda \( (\alpha_2\beta_2^{145\text{His}}) \), the substitution of histidine for the penultimate tyrosine of the \( \beta \) chains would be expected to alter, if not disrupt, the normal conformation of the COOH-termini in the \( \beta \) chains, thus impairing formation of a normal deoxy structure. However, the extent to which perturbations in the \( \beta \) chains can influence the structure, and therefore presumably the function, of the \( \alpha \) chains is not clear. Similarly, it is impossible to judge from Perutz's model whether or not the introduction of an imidazole at position 145 \( \beta \) would influence the intrinsic ligand binding properties of the \( \beta \) heme sites in either the oxy- or the deoxyhemoglobin quaternary structure.

In general, most mathematical treatments of the behavior of abnormal hemoglobins have assumed that the \( \alpha \) and \( \beta \) chains are identical, and that the amino acid modifications influence only the transition between oxy and deoxy structures and not the intrinsic ligand binding properties of the heme sites within these structures (7, 8). In terms of the allosteric model of Monod et al. (8, 9), this is interpreted as a change in the equilibrium constant, \( L \), describing the hypothetical transition from the deoxy (T) to the oxy (R) conformation as opposed to a change in \( K_T \) or \( K_R \), the intrinsic binding constants of these two conformations. Recently, some of these intrinsic constants have been measured.
with the result that the α and β chains can no longer be considered equivalent (10-13). It should therefore be possible to determine the extent to which the substitution of histidine for tyrosine at position 145 β influences the properties of the α and β chains within the deoxy andoxy conformations and also the extent to which this mutation alters the over-all transition during ligand binding.

MATERIALS

Purified samples of hemoglobins A and Bethesda obtained from the same patient were a generous gift from Dr. Franklin Bunn, Thorndike Memorial Laboratory, Boston City Hospital, Boston, Massachusetts. The samples of hemoglobin A obtained during the purification of hemoglobin Bethesda were used as controls (6). Concentrations in terms of heme were determined spectrophotometrically by converting the hemoglobin samples to the CO form and then using the extinction coefficients of Banerjee et al. (14). Solutions of n-butyl isocyanide, CO, O₂, and deoxyhemoglobin were obtained and prepared as previously described (10). IHP, and bis-tris were obtained from Sigma, Calbiochem, and Aldrich, respectively. The Manox brand of sodium dithionite was a gift from Holdman and Harden, Miles Platting, Manchester, England.

METHODS

All static measurements presented were made with a Cary 15 spectrophotometer. All kinetic measurements were made using the stopped flow apparatus and data collection system described by Gibson (15) and DeSa and Gibson (16). Analysis of the CO and n-butyl isocyanide binding data in terms of two independent kinetic components was obtained by fitting the observed time courses to the sum of two exponentials

\[ Y = A_1e^{-k_1t} + A_2e^{-k_2t} \]  

under conditions where the concentration of ligand was at least 4 times the total concentration of heme (pseudo first order conditions). The sedimentation equilibrium experiments were carried out simultaneously in a four-hole rotor using a Beckman model E analytical ultracentrifuge equipped with scanning absorption optics.

RESULTS

Oxygen Dissociation

The dissociation of the first molecule of oxygen from saturated oxyhemoglobin may be measured by mixing solutions of hemoglobin equilibrated with various concentrations of oxygen with buffer equilibrated with 1 atm of carbon monoxide. Olson et al. (11) found that this replacement reaction can be described accurately by the following scheme which incorporates the differences between the α and β chains

\[ \alpha O_2 \xrightarrow{k_{\alpha}} \alpha + O_2 \quad \beta O_2 \xrightarrow{k_{\beta}} \beta + O_2 \]

\[ \alpha CO \quad \beta CO \]

The various rate constants apply only to the last step in ligand binding (\( Hb_4X + X \leftrightarrow Hb_4X \)) and are determined under conditions where the hemoglobin molecules contain a minimum of three bound ligands (11). A series of replacement reactions were performed with hemoglobin Bethesda. The values of the parameters shown in Table I were obtained using the fitting procedure described by Olson et al. (11) and are virtually identical with those obtained previously with oxyhemoglobin A.

The reaction of oxyhemoglobin with dithionite is more complicated and exhibits a time course which reflects not only the rate of release of the first \( O_2 \) molecule but also the rate of release of subsequent \( O_2 \) molecules from the partially saturated intermediates generated during the reaction. Two limiting cases may be distinguished: (a) the rates of dissociation from the partially saturated intermediates are statistically related to the rates of dissociation from saturated oxyhemoglobin; (b) the rates of dissociation from all partially saturated intermediates are much larger than the rates of dissociation from saturated oxyhemoglobin. In the first case, the observed initial rate (in terms of the data given in Table I) will be \( (k_a + k_p) / 2 \) and the amplitudes of the two chain components will be equal. In the second case, the initial rate will be \( 2(k_a + k_p) \) and the amplitudes of the components will be \( k_p / (k_a + k_p) \) and \( k_a / (k_a + k_p) \). These two cases represent no interaction and infinite subunit interaction, respectively. The first case may be simulated experimentally by adding carbon monoxide to the dithionite solution. Since the intermediates formed during the reaction should contain, under appropriate conditions, a minimum of three bound ligands (11), the time course should resemble that calculable from the values of \( k_a \) and \( k_p \) given in Table I.

The experiments illustrated in Fig. 1 show that hemoglobin Bethesda reacts with dithionite at about the rates and with the time course corresponding to (a) described above. The unfilled symbols in Fig. 7 show that the time course is virtually unaffected by changing the buffer from 0.05 M bis-tris to 0.05 M Pi. Such a change modifies the behavior of hemoglobin A by substantially increasing the apparent first order rate constant at 50% deoxygenation from about 25 to 40 sec⁻¹, and by altering the time course to accentuate its autocatalytic character. Thus oxyhemoglobin Bethesda does not exhibit cooperative oxygen release, a conclusion which is predicted by the equilibrium studies of Bunn (6). This result is further confirmed by the experiment using a dithionite solution saturated with CO. Since (a) described above applies, little change is produced by the presence of CO in the dithionite solution (● in Fig. 1).

<table>
<thead>
<tr>
<th>Table I</th>
<th>Replacement of oxygen from saturated oxyhemoglobin by carbon monoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>( k_a )</td>
</tr>
<tr>
<td>A</td>
<td>13.1 ± 0.8</td>
</tr>
<tr>
<td>Bethesda</td>
<td>13.2 ± 0.4</td>
</tr>
</tbody>
</table>

The rate constants for the dissociation of oxygen from saturated oxyhemoglobin Bethesda were calculated according to the scheme given in the text using the procedures described by Olson et al. (11). The values for HbA are taken from Reference 11. The reaction conditions for both types of hemoglobin were 0.05 M \( \Gamma_1 \), pH 7.0, 20°C.
The concentration of Hb-Bethesda was 10 μM before mixing and observations were made at 430 nm through a 2-cm path length cell in the stopped flow apparatus. All reactions were carried out at pH 7.0, 20°. □ and ▲, reaction of HbO₂ with dithionite in the absence of CO; ●, reaction of HbO₂ with a dithionite solution containing 0.92 mM CO. Conditions: ●, 0.05 M Pi; □, 0.05 M bis-tris; ▲, 0.05 M Pi. ---, expected time course for the reaction of stripped oxyhemoglobin A with dithionite in 0.05 M bis-tris, pH 7.0.

The values shown represent apparent second order rate constants. In those reactions which exhibited accelerating time courses, the initial rates are given. In those which exhibited biphasic time courses, the data were fitted to Equation 1, and the resultant values of the exponents are shown. The values for HbA were derived from the data in Fig. 2, and the values for Hb- Bethesda from the data in Fig. 3. The reaction conditions are given in the legends to these figures.

<table>
<thead>
<tr>
<th>Hemoglobin sample</th>
<th>Condition</th>
<th>Number of components</th>
<th>$k_{fast}$</th>
<th>$k_{slow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>pH 7.0</td>
<td>1</td>
<td>$2.1 \times 10^{-6}$ M⁻¹ sec⁻¹</td>
<td></td>
</tr>
<tr>
<td>Bethesda</td>
<td>pH 6.0</td>
<td>2</td>
<td>61</td>
<td>65</td>
</tr>
<tr>
<td>Bethesda</td>
<td>pH 6.0 + IHP</td>
<td>1</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Bethesda</td>
<td>pH 7.0</td>
<td>2</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>Bethesda</td>
<td>pH 7.0 + IHP</td>
<td>1</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Bethesda</td>
<td>pH 8.0</td>
<td>2</td>
<td>72</td>
<td>82</td>
</tr>
<tr>
<td>Bethesda</td>
<td>pH 8.0 + IHP</td>
<td>2</td>
<td>32</td>
<td>35</td>
</tr>
</tbody>
</table>

Carbon Monoxide Binding—A comparison of the time course of carbon monoxide binding to hemoglobins A and Bethesda is shown in Fig. 2 and Table II. The sample of hemoglobin A used in these experiments was obtained during the isolation of hemoglobin Bethesda, and the results are in excellent agreement with previously published data. The most striking finding in these experiments is the extremely rapid rate of CO binding to deoxy hemoglobin Bethesda. Even in the presence of saturating amounts of IHP, the rate of binding remains greater than that observed for stripped deoxyhemoglobin A (Table II). In the case of stripped hemoglobin Bethesda, the time course for the CO reaction is biphasic and exhibits wave length dependence (Figs. 3 and 4). The rate of binding to the rapid component is similar to that observed for $k_{fast}$ isolated chains, and other forms of rapidly reacting hemoglobin (7, 17). Even the rate of binding to the slow component is 5 times greater than that observed for hemoglobin A. This result indicates a lack of cooperative binding and further suggests that the conformation of deoxyhemoglobin Bethesda is similar to that of liganded hemoglobin A. The addi-
tion of IHP can then be thought of as modifying this structure to a more deoxy-like conformation so that, in its presence, more normal kinetic behavior is observed (a slightly accelerating time course with a rate of the order of $2 \times 10^5 \text{M}^{-1} \text{sec}^{-1}$).

The pH dependence of the time course of CO binding to both stripped and IHP-treated hemoglobin Bethesda is shown in Fig. 3 and Table II. In the absence of phosphates, the rates of both the fast and the slow components exhibit a monotonic increase as the pH is raised, with the rate of the fast component at pH 8.0 being somewhat greater than that reported in partial photolysis experiments with hemoglobin A (17). In the presence of IHP, the time course at pH 6.0 is similar to that observed at pH 7.0, and both time courses exhibit homogeneous, slowly reacting behavior. However, at pH 8.0, even with excess IHP, the time course is biphasic, consisting of a rapid component with a rate about 15 times faster than that observed with stripped hemoglobin A and of a slow phase with a rate about equal to the initial rates observed under similar conditions at pH 6 and 7 (Table I). Apparently IHP is unable to convert deoxyhemoglobin Bethesda completely to a slowly reacting form at pH 8.0.

The wave length dependence of the time courses of CO binding to the various hemoglobin samples is shown in Fig. 4. As previously described (16), the time course for stripped hemoglobin A exhibits no wave length dependence, but on the addition of IHP to the same sample, evidence is observed for a rapid spectral component with an isosbestic point at about 424 nm and a slower component with an isosbestic point at about 426 nm (Fig. 4C). In contrast, spectral heterogeneity is observed for stripped hemoglobin Bethesda (Fig. 4A), but, in this case, the rapid component is isosbestic at about 426 nm and the slow component at about 424 nm, and both rates are much greater than those seen with IHP-treated hemoglobin A. The positions of the isosbestic points for the corresponding CO binding reactions with the isolated chains are 425 nm for the $\alpha$ chains and 427 nm for the $\beta$ chains. Since hemoglobin Bethesda exhibits noncooperative, chain-like behavior under these conditions, the more rapidly reacting spectral component seen with stripped Bethesda can be tentatively designated as representing $\beta$ chains and the more slowly reacting spectral component seen with stripped Bethesda can be tentatively designated as representing the $\alpha$ chains. The addition of IHP to deoxyhemoglobin Bethesda at pH 6 or 7 abolishes this spectral heterogeneity and causes the observed time courses at the appropriate wave lengths to resemble those of stripped hemoglobin A (Fig. 4B).

n-Butyl Isocyanide Binding—The reaction of deoxyhemoglobin Bethesda with n-butyl isocyanide was studied in an attempt to measure the differential properties of the $\alpha$ and $\beta$ chains. As expected (10), the time course of n-butyl isocyanide binding was biphasic and wave length dependent (Fig. 5). However, the shape of the time course is widely different from that of hemoglobin A. The percentage of the absorbance change due to the fast component was roughly 50 compared with the value of 23 observed for hemoglobin A under similar conditions, and the slow rate is about 8 times greater than that seen with hemoglobin A (13). The fast and slow components observed with hemoglobin Bethesda can be isolated spectrally near the 435 nm isosbestic point (Fig. 5B). This spectral dependence suggests that the fast component represents $\beta$ chains and the slow component, $\alpha$ chains, since in this spectral region, for both isolated chains and those within deoxyhemoglobin A, the $\beta$ subunits are isosbestic at 435 nm (wave lengths longer than that static isosbestic...

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R. Cassoly and Q. H. Gibson, manuscript in preparation.
2,3-diphosphoglycerate (added to hemoglobin solution); A, 0.05 molar isocyanide after mixing was 460 μM. Solvent conditions: • 0.05 M bis-tris; O, 0.05 M Pi; △, 0.05 M bis-tris plus 300 μM 2,3-diphosphoglycerate (added to hemoglobin solution); Δ, 0.05 M Pi plus 123 μM IHP (added to hemoglobin solution).

**Fig. 6.** The effect of phosphates on the reaction of deoxyhemoglobin Bethesda with n-butyl isocyanide. The reaction conditions are identical with those in Fig. 5B, except that the concentration of isocyanide after mixing was 460 μM. Solvent conditions:

- • 0.05 M bis-tris;
- O, 0.05 M Pi;
- △, 0.05 M bis-tris plus 300 μM 2,3-diphosphoglycerate (added to hemoglobin solution);
- Δ, 0.05 M Pi plus 123 μM IHP (added to hemoglobin solution).

**Table III**

**Kinetic parameters for reaction of deoxyhemoglobin Bethesda with n-butyl isocyanide in 0.05 M bis-tris, pH 7.0, 20°C**

The deoxy kinetic constants have been designated as those which represent the binding of the first isocyanide molecule to tetrameric hemoglobin, and the liganded constants as those which represent the binding of the last isocyanide molecule (13). The symbols i' and i represent association and dissociation velocity constants, respectively. The values of the parameters for HbA were obtained previously (10, 13). The values for Hb-Bethesda were obtained as described in the text.

<table>
<thead>
<tr>
<th>Hemoglobin sample</th>
<th>Chain</th>
<th>Free reaction</th>
<th>Liganded reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(HbA + X = HbAX)</td>
<td>(HbA+1 + X = HbAX)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i'</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i'</td>
<td>i</td>
</tr>
<tr>
<td>A</td>
<td>α</td>
<td>0.4</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>44.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Bethesda</td>
<td>α</td>
<td>18</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>130</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.32</td>
<td>0.19</td>
</tr>
</tbody>
</table>

The points, while the α subunits are isosbestic at 433 to 434 nm (wave lengths shorter than the static isosbestic point) (10, 13). The relative rates of binding to the two spectral components are also consistent with this idea since, under all conditions at pH 7, the β chains react more rapidly with n-butyl isocyanide (10, 13).

The effect of phosphates on the time course for the binding of n-butyl isocyanide to hemoglobin Bethesda is shown in Fig. 6. Inositol hexaphosphate exerts the greatest effect, and, in its presence, the slow rate is reduced to a value which is only about 2 times greater than that observed for the slow component in experiments with stripped hemoglobin A. In all cases, the time course can be represented by the sum of two exponentials (Equation 1). The dependence on ligand concentration of the relative amplitudes of the two spectral components and of their observed first order rates (the exponents in Equation 1) is shown in Fig. 7 and compared with that observed previously with stripped hemoglobin A (13).

If the two components react independently, the isocyanide concentration dependence of k_f and k_s (Equation 1) should be given by

\[ K_f = i'_f (BIC) + i_f, \quad K_s = i'_s (BIC) + i_s \]  

where i' and i represent association and dissociation velocity constants, respectively, and BIC represents n-butyl isocyanide. If the two components have equal affinities for this ligand, the percentage of the fast or β chain component should be independent of ligand concentration and would simply represent that proportion of the absorbance change which is due to the fast component. As shown in Fig. 7, Equation 2 does, indeed, appear to describe the ligand concentration dependence of the fast and slow rates, and, in sharp contrast to hemoglobin A (--- in Fig. 7), the percentage of the fast component is independent of ligand concentration and nearly equal to 50 at 428 nm, as one would expect if the two components represented the individual chains (13). Thus, it appears that the α and β chains within stripped deoxyhemoglobin Bethesda are reacting independently. A detailed interpretation of the results of similar experiments with hemoglobin A has been presented elsewhere (13), and for the present purpose it is sufficient to say that the marked dependence of the percentage of fast component on ligand concentration seen with hemoglobin A indicates strong subunit interaction.

A comparison of the n-butyl isocyanide binding properties of the α and β chains within stripped deoxyhemoglobins A and Bethesda is given in Table III. The values of i' and i for the reaction of the first isocyanide molecule (and also subsequent molecules, since the system is noncooperative) with deoxyhemoglobin Bethesda were obtained from measurements of the time course of the carbon monoxide replacement reaction (10). These values are identical with those obtained from the intercepts in Fig. 7, which again indicates the absence of subunit interaction. Similarly, the values of i'α and i'β obtained from the slopes in Fig. 7 are almost equal to the values of i'α and i'β previously determined for the reaction of hemoglobin A with the last molecule of n-butyl isocyanide.

**Properties of Deoxyhemoglobin Bethesda**—It has been shown previously that inositol hexaphosphate equilibrates rapidly with both unliganded and fully liganded hemoglobin A (18). For example, in CO binding experiments the full effect of IHP is still observed even when, instead of adding this phosphate directly to the hemoglobin solution, it is added to the CO solution. Thus we expected that the effect of IHP on CO binding to hemoglobin Bethesda could also be measured by simply adding it to the CO solution. However, the situation is more complicated than was first anticipated. When IHP was present only in the CO solution, about 60% of the ligand binding sites still remained in a rapidly reacting form. When IHP was then added to the hemoglobin solution and the experiment repeated, the time course of CO binding was no longer biphasic but gently accelerating with an initial rate of about 3 \times 10^5 M^{-1}sec^{-1} (Table II, Figs. 3 and 8). Thus, either IHP does not equilibrate rapidly with hemoglobin Bethesda or the conformational change induced by this phosphate is slow.

A further indication that IHP was producing a rather drastic
Fig. 7. The ligand concentration dependence of the amplitudes and rates for the fast and slow components observed in the reaction of deoxyhemoglobin Bethesda with n-butyl isocyanide (BIC). The values for the parameters were obtained from fits of the various time course to Equation 1. The reaction conditions are given in Figs. 5 and 6. •—•, data for stripped Hb-Bethesda in 0.05 M bis-tris, pH 7.0; •—•, data for IHP-treated Hb-Bethesda in 0.05 M Pi, pH 7.0; •—•, expected dependencies for stripped HbA in 0.05 M bis-tris, pH 7.0 (13).

effect on the deoxyhemoglobin conformation came from a comparison of the total absorbance changes observed in the mixing experiments shown in Fig. 8. Taking the total change observed in the absence of IHP as a reference, the addition of IHP to the CO solution resulted in a 10% increase in the absorbance change observed at 432 nm. When IHP was then added to the hemoglobin solution, the total absorbance change became 20% greater than that observed initially. Since IHP does not significantly alter the spectrum of HbO(CO)₄, these changes must be attributed to an alteration in the Soret spectrum of unliganded hemoglobin Bethesda. Therefore, it seemed logical to measure the rate and the extent of this change by mixing deoxyhemoglobin Bethesda with deoxygenated buffer containing IHP, and the results of several such experiments are shown in Fig. 9. The time course of the change in the deoxyhemoglobin spectrum is markedly biphasic at all wave lengths. The rate of the fast phase is independent of both heme and IHP concentration, indicating that the rate-limiting step is first order. However, the extent of the over-all reaction and the relative amplitude of the slow phase do depend on these parameters. If the reciprocal of the absorbance change is plotted versus time, a linear relationship is observed for the slow phase of the reaction, which suggests that some type of second order association phenomenon is occurring. Since IHP binds to both liganded and deoxyhemoglobin with a stoichiometry of 1 mole bound per mole of tetramer (18), the simplest interpretation of the slow phase shown in Fig. 9 is that it is due to the association of deoxy dimers to form trimers which subsequently react with IHP. This would imply a significant amount of subunit dissociation at the heme concentrations used in these mixing experiments. In order to test this idea, sedimentation equilibrium runs were performed with deoxyhemoglobin Bethesda solutions containing various amounts of IHP, and the
The natural logarithm of the absorbance at 405 nm ($A_{405}$) is plotted versus the square of the distance from the center of the rotor ($X^2$). In each case the initial concentration of heme was 8 μM in a solution of 0.1 M Tris, pH 7.0, containing 2 mg per ml of dithionite. Equilibrium was achieved by spinning overnight at 94,000 × g at 20°C. The slope of these curves is proportional to the weight average molecular weight. The upper and lower dotted lines (--- in each panel) represent expected curves if the solutions contained either all tetramers or all dimers, respectively. The positions of these lines were fixed so that they intersected the ordinates at the same positions as the observed data, and thus were not calculated for the initial starting conditions. A, data for deoxyhemoglobin Bethesda alone; B, carboxyhemoglobin Bethesda; C, deoxyhemoglobin Bethesda plus 12.3 μM IHP; D, deoxyhemoglobin Bethesda plus 123 μM IHP.

results are shown in Fig. 10. The HbCO solution was prepared by taking the stock deoxyhemoglobin solution and gently passing carbon monoxide over it. The two arrows in these panels indicate the heme concentration before (upper arrows, about 8 μM) and after mixing (lower arrows, about 4 μM) in most of the ligand binding experiments which have been described earlier. It is clear from Fig. 10A that a substantial portion of deoxyhemoglobin Bethesda is dissociated at the heme concentrations shown. A comparison with Fig. 10B indicates that the extent of dissociation is roughly the same as that observed for liganded hemoglobin Bethesda. The two lower panels show that the addition of IHP decreases the extent of dissociation and that, if added in excess, only tetrameric hemoglobin is observed.

The static difference spectra of IHP-treated Hb-Bethesda versus stripped Hb-Bethesda are shown in Fig. 11. The largest changes were observed for deoxyhemoglobin Bethesda in bis-tris at pH 6 and 7. These difference spectra are virtually identical with those observed upon mixing isolated α and β chains to form native hemoglobin A (19) and with those observed upon the reassociation of deoxy dimers to form tetrameric hemoglobin A (20, 21). This result, coupled with the rapid rates of CO and n-butyl isocyanide binding and the extensive degree of subunit dissociation, suggests that stripped deoxyhemoglobin Bethesda exists in solution in a liganded like conformation. In agreement with the CO binding results in Fig. 3, the smaller absorbance change at pH 8.0 appears to reflect the inability of IHP to produce a complete conversion to a normal deoxy structure at this pH. In contrast, the smaller absorbance change observed in 0.1 M Tris, pH 7.0, can be interpreted as due to the inorganic phosphate causing a partial conversion to the normal deoxy conformation so that, in this case, the initial hemoglobin solution already contains substantial proportions of deoxyhemoglobin A-like conformations.

**DISCUSSION**

Hemoglobin may be considered to exist in at least two distinct conformational states which, in terms of crystallographic studies, are usually designated as the deoxy and liganded structures (1). When discussing the properties of hemoglobin A, it is conventional to assign those kinetic constants which describe the binding of the first ligand molecule (Hb4 + X = Hbx X) to the deoxy structure and those which describe the binding of the last molecule (Hbx X + X = Hbx X X) to the liganded structure. The values of the rate constants for these two reactions are usually determined by different types of kinetic experiments; the parameters for the deoxy conformation are measured by direct mixing experiments (deoxyhemoglobin plus ligand (13)), while those for the liganded conformation are derived from CO replacement and partial photolysis experiments (10). As seen in Tables I and III, the reactions of liganded hemoglobins A and Bethesda cannot be distinguished by the CO replacement reactions that were examined. This is true not only of the gross behavior but also of that of the individual chains. Thus, the substitution of histidine for tyrosine 145 β has no effect on the binding properties of either the α or the β chains within the liganded conformation. It should be pointed out that the behavior of the isolated chains is measurably different from that of the chains within tetrameric liganded hemoglobin (10, 11), so that we are unable to tell whether or not this mutation alters the properties of the isolated β chains.

An entirely different picture emerges when the behavior of deoxyhemoglobins A and Bethesda are compared. Under all circumstances the reactions of deoxyhemoglobin Bethesda resemble more closely those of the liganded conformation than those of deoxyhemoglobin A. The reaction with n-butyl isocyanide is most conveniently analyzed in terms of the individual chains, and the results in Table III show that the reactions of the α and β chains within deoxyhemoglobin Bethesda are similar, within a factor of two, to those of the chains within liganded hemoglobin A. Roughly similar results were observed when the carbon monoxide binding reaction was studied. At low pH and ionic strength there is clear evidence for two distinct spectrophotometric components with rates differing 5- to 10-fold (Fig. 3 and Table II). Analogy with results obtained with n-butyl isocyanide and the positions of the isosbestic points for the two phases in carbon monoxide binding would suggest that the faster reacting component represents β chains and the slower component α chains. However, these kinetic components appear to be unequally influenced by increasing pH, which increases both the rate and the proportion of the rapid phase. The addition of IHP markedly changes the time course of CO binding to hemoglobin Bethesda. At low pH the rapid phase is abolished, and the remaining slow phase is 3 to 4 times faster than that observed for hemoglobin A under similar conditions (Fig. 2). At the same time there is a change in the absorption spectrum of deoxyhemoglobin Bethesda from that characteristic of a rapidly reacting hemoglobin, with a depressed Soret maximum, towards that of normal deoxyhemoglobin A (Fig. 11). Corresponding
alterations are observed in the difference spectrum which change, on the addition of IHP, from that corresponding to isolated chains to an appearance similar to that of stripped hemoglobin A (Fig. 4).

The changes in the Soret absorption spectrum of deoxyhemoglobin Bethesda produced by the addition of IHP are within the reach of direct study by the stopped flow method. As shown in Fig. 9, these changes occur in two phases which are widely separated in rate. The slower of these is independent of IHP concentration (assuming stoichiometric excess) but varies in extent and rate as the concentration of hemoglobin is varied. The amplitude of the slow phase, as a proportion of the total absorbance change, decreases with increasing protein concentration while the initial rate of this phase increases. This behavior is consistent with subunit aggregation. This interpretation is fully supported by the ultracentrifuge experiments which show that deoxyhemoglobin Bethesda has about the same tetramer to dimer dissociation constant as liganded hemoglobin A and Bethesda (Fig. 10).

Since IHP binds to hemoglobin with a stoichiometry of one mole per mole of tetramer, the slow phase of the Soret change produced by IHP may be interpreted as due to the formation of slowly reacting tetramers from noncooperative, rapidly reacting dimers.

The fast phase of the Soret change exhibits quite different characteristics. The rate of this phase is independent of hemoglobin and IHP concentrations, and therefore, it is necessary to postulate some type of first order isomerization reaction preceding the binding of IHP, and that the product of this reaction is a deoxyhemoglobin conformation with a normal (HbA type) Soret absorption spectrum. In agreement with previous work (18), the lack of dependence on IHP concentration of the rate for either phase show that this organic phosphate equilibrates rapidly with tetrameric hemoglobins. Thus, the reactions involved in the binding of IHP to deoxyhemoglobin Bethesda may be written

\[
\frac{2 \text{Hb}_2^*}{k_1} \xrightarrow{k_2} \frac{\text{Hb}_4 + \text{IHP}}{k_4} \xrightarrow{k_3} \frac{\text{Hb}_4(\text{HP})}{K_{\text{HP}}}
\]

The rate of the conversion from the rapidly reacting forms with a depressed Soret absorption spectrum (starred species) to the slowly reacting forms (unstarred species) is governed by the rate constants involving dimers, \(k_1\) and \(k_2\), which are similar to those observed by Andersen et al. (20) and Nagel and Gibson (22) for liganded hemoglobin A, and the observed rapid first order rate corresponding to \(k_3\) and \(k_4\). The extent of the reaction is determined by these constants and the IHP equilibrium binding constant, \(K_{\text{HP}}\). The dotted arrows represent an alternative pathway for the binding of IHP to HbA*. In this pathway the isomerization process would follow the binding of IHP. However, since IHP has been shown to equilibrate extremely rapidly with both liganded and deoxyhemoglobin (18), the observed fast phase would primarily reflect the rate of the \(\text{HbA}^*(\text{IHP}) \rightleftharpoons \text{HbA}(\text{IHP})\) isomerization reaction. Thus, the two pathways are virtually indistinguishable, but there is no doubt that the observed fast phase represents a first order conformational change.

The scheme, as presented, was able to fit the results of the IHP binding experiments with a fair degree of success and yielded the results shown in Fig. 12. The parameters obtained in this manner agree well within the limits suggested by the ultracentrifuge experiments but, unfortunately, do not exactly reproduce more detailed equilibrium titrations. However, the suggestion arising from the values of these parameters (given in the legend to Fig. 12) is that the species \(\text{Hb}_4\) is sparsely populated in stripped deoxyhemoglobin Bethesda, which appears to consist primarily of \(\text{Hb}_2^*\).
and Hb4*. Thus, as also suggested by the ligand binding results, deoxyhemoglobin Bethesda in bis-tris may be thought of as having the conformation and properties of liganded hemoglobin.

Although the idea that deoxyhemoglobin may exist in more than one form is not new (23), rather few direct observations of the interconversion of Hb4* and Hb4 have been made. In part, this is due to the rapidity of the rate of this transition which has been estimated by Gibson and Parkhurst (24) to be at least $10^6$ sec$^{-1}$ at pH 7.0, 20°C for human hemoglobin A in 0.1 M Pi. If, in general, hemoglobins in the liganded conformation bind anions less strongly than in the deoxy conformation, transitions from Hb4* to Hb4 must occur on the addition of compounds such as IHP to deoxyhemoglobin solutions. Indeed, in unpublished experiments, changes in the Soret absorption spectrum of deoxyhemoglobins Chesapeake (25), Hiroshima (26), desArg 141 α (2), 4 desHis 146 α (2), and the cyanomet valence hybrids (27) have been observed on the addition of IHP to these compounds. However, hemoglobin Bethesda is the first example in which the rate of this transition has been accessible to direct stopped flow methods.

In summary, it is quite clear that the substitution of histidine for tyrosine 145 α drastically alters the ability of hemoglobin to form a normal deoxy structure and, therefore, to exhibit cooperativity. The importance of tyrosine 145 α in maintaining the deoxy quaternary structure has been discussed at length by Perutz and co-workers (1, 3), and it is certainly probable that the protonated, if not also the unprotonated, form of the imidazole group is incapable of forming a stable conformation in the hydrophobic pocket between the F and H helices. Thus, a priori, a normal deoxy structure would not be expected. However, this mutation does not appear to alter the intrinsic reactivities of the heme sites in the liganded conformation, but rather, it inhibits the quaternary change to a deoxy structure in such a manner

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