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Biophysics

Self-association of hemoglobin β^{SH} chains is linked to oxygenation

(protein association/ligand binding/thermodynamics/equilibrium gel permeation)

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ABSTRACT **Self-association of unliganded β^{SH} chains into tetramers ($4\ \beta_1 \rightharpoonup \beta_4$) is experimentally found to be energetically less favorable ($\Delta G^0 = -19.05 \pm 0.30$ kcal) than the corresponding oligomerization of fully oxygenated chains ($4\ \beta_1 X \rightharpoonup \beta_4 X_4$; $\Delta G^0 = -22.45 \pm 0.35$ kcal). Hence the tetramers must bind oxygen with a higher affinity than that of dissociated chains. Calculations are presented showing why this affinity difference is not easily detected. The linkage is in a direction opposite to that exhibited by normal hemoglobin A, in which oligomerization of high-affinity unliganded dimers ($2\ \alpha\beta \rightharpoonup \alpha_2\beta_2$) leads to tetramers with decreased oxygen affinity. In contrast, the oligomerization of high-affinity, unliganded β^{SH} chains leads to tetramers with even higher affinity. The results imply the existence of at least two conformational states for β chains.**

Effects of inositol hexaphosphate on β chain association were investigated. Inositol hexaphosphate was found to have no measurable effect at pH 7.4, in contrast to pH 7 where very pronounced effects have been observed. Some theoretical aspects of the linkages are presented and the relationship of the findings to concepts of structural transition and allosteric regulation is discussed.

In contrast to the β chains, self-association of α chains into dimers was found to occur with the same free energy in both unliganded and fully oxygenated states. Thus, the self-association of α chains is not linked to oxygenation.

Isolated α and β chains of human hemoglobin bind oxygen with high affinity similar to that of myoglobin (see ref 1). They also self-associate to form dimers and tetramers (2). However, in contrast to the heterogeneous tetramers of normal hemoglobin ($\alpha_2\beta_2$), the homogeneous chains have not been found to exhibit cooperativity nor to have measurably altered oxygen affinities as a result of oligomerization (3). Dissociated $\alpha^1\beta^1$ dimers also do not exhibit these latter properties to a significant degree (4–8). Assembly of high-affinity unliganded $\alpha^1\beta^1$ dimers, however, (or a stoichiometric combination of separated α and β chains) into unliganded tetramers results in a species of greatly lowered oxygen affinity and high cooperativity (see ref 8). This decrease in oxygen affinity accompanying assembly of chains into tetramers constitutes a theromdynamic constraint forming the basis of cooperative events. This constraint is released during the successive oxygenation steps in parallel with the quaternary structural transition inferred from x-ray crestallographic determinations (9, 10). The x-ray structural results and the thermodynamic characterization of subunit assembly and oxygen binding all suggest the necessity of $\alpha^1\beta^2$-type contacts within a tetrameric molecule as a requirement for the particular type of cooperativity observed in normal human hemoglobin (see ref. 11). However, the energetic and conformation states of the various subunits and the quaternary structures assumed during the intermediate stages of oxygenation are still unknown. Consequently, detailed studies on the structural and thermodynamic states that can be assumed by the individual components of the hemoglobin system continue to be of considerable interest.

From x-ray crystallographic analysis Perutz and Mazzarella (12) concluded that the quaternary structure of oxygenated chains β_4X_4 is similar to that of the "constrained" deoxy form of normal hemoglobin $\alpha_2\beta_2$, (i.e., a "T-like" structure). The latter species has a free energy of subunit association that is more negative by 6–8 kcal than that of the ("R-like") oxygenated molecule (7, 13). Corresponding structural studies with unliganded β_4 chains have not been reported to date. It is nevertheless of interest to determine the relationship between energetics of assembly in both oxygenated and unliganded chains to see whether oligomerization is oxygenation-linked as in the case of normal hemoglobin A.

In recently reported studies, we used a combination of molecular sieve chromatography and calorimetry to define the stoichiometries and thermodynamics of self-association for isolated α and β chains in their fully oxygenated states (2, 14). Here we present new results on the self-association of these chains in the unliganded state. For these studies we have used the technique of equilibrium gel permeation by direct optical measurement in a gaseous atmosphere-controlled system using a computer-controlled single photon counting spectrophotometer, described elsewhere (ref. 15, unpublished data). With this instrument the weight-average partition cross sections for unliganded chains have been determined as a function of protein concentration and the resulting dissociation curves analyzed for stoichiometry and equilibrium constants. For comparison, (and in confirmation of previous results) we have also determined dissociation curves under oxygenated conditions with the same apparatus. The results clearly establish the oligomerization of β chains to be oxygenation-linked. An additional unexpected result is that the linkage operates in a direction opposite to that of normal hemoglobin, i.e., the oxygenated chains, βX, have the greater tendency to oligomerize as compared with the unliganded chains. The possible effects of inositol hexaphosphate (Ins-P_6) upon these reactions has also been explored.

METHODS

Hemoglobin A was purified from freshly drawn blood, and chains were subsequently prepared as described (2). Protein samples were stored in liquid nitrogen prior to their use. Experiments were at 21.5°. Unless otherwise noted, a "standard buffer" was used throughout, consisting of 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2 EDTA at pH 7.4.

Weight-average partition cross sections, $\bar{\xi}_w$, were determined by the equilibrium gel permeation method (15) with a gel-packed quartz flow cell (0.2-ml bed volume, 0.5-cm pathlength)

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Abbreviation: Ins-P_6, inositol hexaphosphate.

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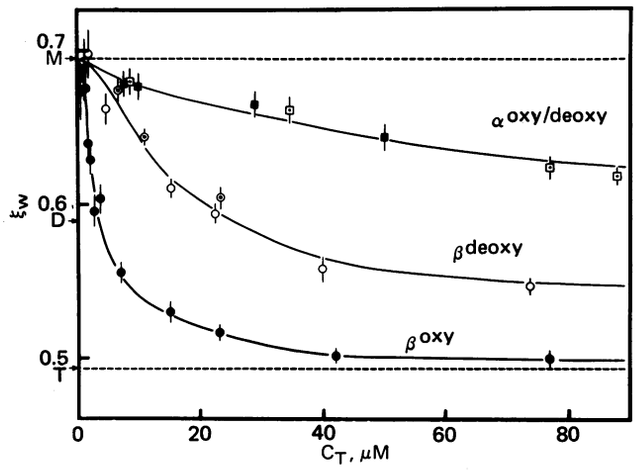


FIG. 1. Weight-average partition cross sections, $\bar{\xi}_w$, against total protein concentration, C_T, for α^{SH} and β^{SH} chains. Solid symbols are measurements under fully oxygenated conditions. Open symbols are for unliganded chains measured under anaerobic conditions in the absence of dithionite. Open symbols with points represent experiments in the presence of 0.1% sodium dithionite. Monomer (M) and tetramer (T) end-points were estimated, respectively, by the partition cross section of myoglobin and by least squares analysis of data to the stoichiometry $4M \rightleftharpoons M_4$ for β chains and $2M \rightleftharpoons M_2$ for α chains (solid curves). The dimer partition cross section (D) was estimated by independent calibration of the gel with standard proteins.

containing Sephadex G-100 (Pharmacia lot 6164) in series with a reference flow cell of 0.5-cm pathlength. The instrument used for these measurements was a computer-controlled single-photon counting spectrophotometer designed specifically for these measurements (15). This instrument has been equipped with an all-glass controllable gaseous atmosphere flow train (unpublished data) that connects the buffer or sample reservoirs and the flow cells in series and terminates with a polarographic oxygen sensor (Beckman electrode 39065). The system permits alternate passage through the measurement cells of samples from either the buffer or protein-solution reservoirs. Gas was equilibrated by purging both buffer and protein-solution reservoirs with an oxygen/nitrogen atmosphere from a tank of fixed composition (Air Products Co.). The O_2 sensor at the end of the flow train was likewise calibrated against known mixtures of O_2/N_2 gases (Matheson) and the results correlated with spectral changes associated with the O_2 binding properties of hemoglobin A. Sodium dithionite (0.1%) was introduced for end-point verification (ref. 8; unpublished data). When a pure nitrogen tank is used, this gas purging method assures complete deoxygenation of the solute species without the addition of extraneous chemical substances, e.g., dithionite (unpublished data). A few measurements were carried out in the presence of dithionite for comparison. Measurements for these studies were made at wavelengths of 220, 240, and 415 nm, as required for optimum detection at the particular concentrations studied. A linear Beers' law relationship was established at each wavelength over the concentration ranges used.

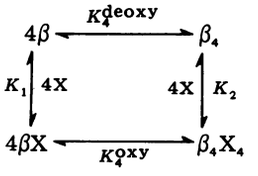
RESULTS AND DISCUSSION

Linkage between Oxygenation and Self-Association. Weight average partition cross sections, $\bar{\xi}_w$, determined as a function of protein concentration C_T (molar heme basis) are shown in Fig. 1 for the unliganded and fully oxygenated chains. The oxygenated β chains are more highly associated (lower $\bar{\xi}_w$) at each concentration than the unliganded chains. Least-squares analyses of these data were done according to procedures described previously (2) in terms of the stoichiometries $4M \rightleftharpoons M_4$ and $4M_1 \rightleftharpoons 2M_2 \rightleftharpoons M_4$, where M represents a β chain in either unliganded or oxygenated form. In both states of ligation a considerably better fit was obtained to the stoichiometry $4M \rightleftharpoons M_4$, as judged by normalized variance of the fits and comparative randomness in the distribution of residuals to the best fit obtained for each stoichiometry. Although the data shown in Fig. 1 for β chains do not, by themselves, cover sufficient range for a definitive assessment of stoichiometry, the observed degree of association is substantially higher than that for formation of dimers alone. Moreover, the least squares fits yielded an estimated tetrameric end-point that agreed closely with that estimated independently by calibration of the gel with standard proteins. These features and the close conformity of data to the expected shape for a tetrameric dissociation curve lend considerable credence to interpretation of the data in terms of a monomer–tetramer equilibrium. These analyses yielded the equilibrium constant: $K_4^{oxy} = 4.95 \times 10^{16}$ liter^3/mol^3 with confidence limits corresponding to one standard deviation of 2.63×10^{16} liter^3/mol^3 and 9.03×10^{16} liter^3/mol^3. The standard free energy ($\Delta G^0 = -RT\ln K_4^{oxy}$) is thus -22.43 ± 0.35 kcal/mol of tetramer. For unliganded chains the equilibrium constant was found to be: $K_4^{deoxy} = 1.52 \times 10^{14}$ liter^3/mol^3 with confidence limits of 0.93×10^{14} liter^3/mol^3 and 2.43×10^{14} liter^3/mol^3, so that the free energy of tetramer formation is -19.05 ± 0.30 kcal/mol of tetramer.

The results obtained for K_4^{oxy} agree very closely with our previous determinations of stoichiometry and equilibrium constant for self-association of oxygenated β chains (2). However, the markedly lower value obtained for K_4^{deoxy} is intriguing in that it indicates a more dissociated form of the β chains under anaerobic conditions.† The effect is reproducible over several different chain preparations, and the unliganded chains, upon reoxygenation, behave like the oxygenated chains that have never been subjected to the deoxygenation procedure.

Thus, a novel and unexpected finding is that the linkage between self-association and oxygenation for β chains is in the opposite direction from that of normal hemoglobin A, in which the liganded form has the greater tendency to dissociate into subunits. The magnitude of the linkage in free energy (3.38 kcal/mol of tetramer) is approximately half that found in normal hemoglobin under identical conditions (8).

In principle, this linkage should also be manifested as a difference between oxygenation curves for monomers and tetramers. The overall linkage relationships may be depicted as:



[1]

$$K_1 = (\beta X)^4/(\beta)^4(X)^4 \qquad K_4^{deoxy} = (\beta_4)/(\beta)^4$$
$$K_2 = (\beta_4 X_4)/(\beta)^4(X)^4 \qquad K_4^{oxy} = (\beta_4 X_4)/(\beta X)^4.$$

We have assumed, for simplicity, here and in subsequent analyses, that β_4 chains bind oxygen noncooperatively. From this linkage scheme and the experimental finding $K_4^{oxy}/K^{deoxy} = 326$, it may be inferred that K_2 must be greater than K_1 by

† That the β chains were in fact unliganded under conditions of these experiments was verified by spectral analyses, and is also indicated by the points obtained in the presence of dithionite, which fall on the same curve as that obtained by atmospherically induced oxygenation.

this same factor. Thus, the free energy of oxygenating four β chains would be 3.38 ± 0.60 kcal more negative when the chains are in the form of tetramers. This difference, amounting to −0.84 kcal/mol of O_2 bound would correspond to a factor of 4.2 [$e^{(0.84/RT)}$] in the molar oxygen concentration at the half-saturation points of binding curves for the two species, i.e., with the curve for monomers lying to the right (increasing pO_2) of the tetrameric curve.‡ The order of these curves is also opposite to that of $\alpha_2\beta_2$ in comparison with $\alpha\beta$ dimers, where the tetramers have the lowest affinity (8).

The difference in positions of saturation curves for monomers and tetramers would be on the order of 0.5 mm Hg under conditions in which most measurements have been made. However, under most experimental conditions only a fraction of this difference can be experimentally observed. An oxygenation experiment carried out at a particular concentration (P_t) of β chains will reflect properties of both curves with increasing contribution from the tetramer curve at high pO_2 values, according to the saturation function:

$$\bar{Y} = \frac{K_1^{1/4}(X) + 4K_1K_4^{oxy}(\beta)^3(X)^4}{1 + K_1^{1/4}(X) + 4K_4^{deoxy}(\beta)^3 + 4K_1K_4^{oxy}(\beta)^3(X)^4} \quad [2]$$

where (X) is the molar concentration of oxygen (related to pO_2 by the Henry's law constant) and β is a unique real positive root of the quartic equation:

$$(P_t) = (\beta)[1 + K_1^{1/4}(X)] + (\beta)^4[K_4^{deoxy} + K_1K_4^{oxy}(X)^4]. \quad [3]$$

Oxygenation curves for β chains have been obtained in this laboratory under the same buffer conditions and temperature of the dissociation studies reported here, at a protein concentration of 50 μM heme. The results were analyzed as a single Langmuir-type isotherm yielding a free energy of −8.56 kcal/mol of heme (P_{50} = 0.27 mm Hg). It can be seen from Fig. 1 that at this protein concentration only about 25% of the dissociation range is available for contribution to the overall isotherm. By varying the protein concentration over the widest feasible range (e.g., down to a few micromolar heme), it may be estimated that shifts in P_{50} of only a few tenths of a millimeter Hg would be exhibited in the oxygenation curves. These shifts would be very near the limits of detectability by present methods. This assessment is consistent with past results showing no noticeable concentration-dependence of β chain oxygenation curves, although systematic efforts to detect such an effect have not been reported to date. Clearly the oxygenation curves are not as sensitive a means of detecting or measuring the linkage as compared with the measurement of shifts in degree of subunit association as a function of ligation state. The latter linkage function may be written:

$$K_T = K_4^{deoxy}[1 + K_2(X)^4]/[1 + K_1^{1/4}(X)]^4. \quad [4]$$

In the present study we have measured the limiting extremes of this function, corresponding to (X) = 0, and (X) = ∞. Measurements of K_T as a function of (X) would provide a test of the assumption that oxygen binding by tetramers may be described by the single constant, K_2.

It is tempting to assert that our experimental findings imply the existence of different conformational states for chains in monomeric compared to tetrameric form or for liganded compared to unliganded chains, but no such simple assignment is possible. It can be seen from the linkage Scheme 1 that the observed difference between K_4^{oxy} and K_4^{deoxy} (and hence between K_1 and K_2) can be explained by the existence of two conformational states among the four species. Further assignment of the differences however cannot be made.

‡ Typical values experimentally obtained for P_{50} on β chains have been in the range 0.25–0.45 mm Hg. The Henry's Law constant pertaining to conditions of this study is 1.8×10^{-6} mol of O_2/mm of Hg.

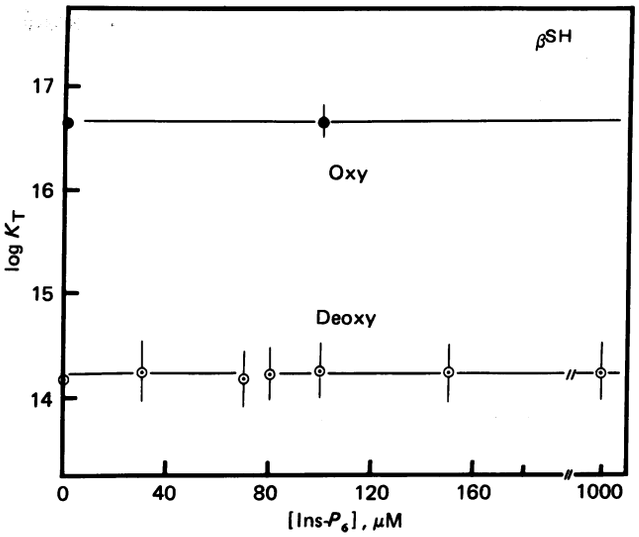


FIG. 2. Equilibrium association constant K_T for tetramer formation against inositol hexaphosphate concentration for oxygenated (solid symbols) and unliganded (open symbols with points) β chains. The oxy data points were taken from ref. 2. Deoxy data points were obtained at a protein concentration of 8 μM heme in the presence of 0.1% sodium dithionite.

Results for self-association of α chains are also shown in Fig. 1. No differences are evident in the partition cross sections for unliganded and fully oxygenated chains. Earlier results (2) show the self-association of oxygenated α chains to be a dimerization reaction.

Measurements with Inositol Hexaphosphate. In a previous study (2) we found no effect of Ins-P_6 upon the self-association of oxygenated β chains, measured under identical buffer and pH conditions with those of this study. Under similar buffer conditions, but at pH 7, Bonaventura *et al.* (16) had reported pronounced effects of high Ins-P_6 concentrations upon the oxygenation curve of β^{SH} chains, shifting it to higher pO_2 values. Even though these studies were carried out under somewhat different conditions, the results taken together suggested that oxygenation and self-association were unlinked, i.e., that the Ins-P_6-induced shift in P_{50} was probably not due to its effect upon the monomer–tetramer equilibrium. Subsequently, pronounced effects of Ins-P_6 upon rates of aggregation of unliganded β^{SH} chains have been reported by McGovern *et al.* (17) under buffer conditions similar to those of this study, but at pH 7 rather than 7.4. In order to obtain a more definitive assessment of the relationship between oxygenation states and aggregation properties we have carried out the experiments described in the previous section, establishing that a linkage does indeed exist. We have also explored effects of Ins-P_6 upon the self-association of unliganded β chains under identical buffer and pH conditions as our previous studies (2). Results are shown in Fig. 2, where the experimentally determined monomer–tetramer equilibrium constant K_T has been plotted against concentration of Ins-P_6 added to the "standard buffer." Over a wide range of concentration the association constant is independent of Ins-P_6 concentration, both for unliganded and fully oxygenated chains. The range was chosen to include the region in which McGovern *et al.* reported a pronounced effect (17). We subsequently were able to reproduce their stopped-flow and spectral results when the pH was lowered from 7.4 to 7.0. Thus, the effect of Ins-P_6 upon self-association of β chains ap-

pears to be strongly pH-dependent in the region near neutrality.

To date the effects of Ins-P_6 upon aggregation and oxygenation properties of β^{SH} chains have been studied under a variety of conditions, leading to a corresponding variety of results. A more extensive and systematic study will be required to define the relationships between the observed effects. The present study establishes that under conditions where aggregation of β^{SH} chains is ligand-linked for oxygen, the self-association reactions are completely insensitive to the presence of Ins-P_6 over a concentration range in which this allosteric effector is known to exhibit dramatic effects in normal hemoglobin.

These results also strongly suggest that appreciable binding of Ins-P_6 to β^{SH} chains does not occur under these conditions. The conclusion may be inferred from the following considerations. We represent the linkage between Ins-P_6 binding and self-association in chains, M, in either unliganded or oxygenated form according to the following generalized scheme

$$\begin{array}{ccc} 4M & \xrightarrow{K_1} & M_4 \\ {\scriptstyle K_2}\uparrow\downarrow{\scriptstyle 4L} & & {\scriptstyle 4L}\uparrow\downarrow{\scriptstyle K_3} \\ 4ML & \xrightarrow[K_4]{} & M_4L_i + (4-i)L \end{array}$$

$$\begin{aligned} K_1 &= (M_4)/(M)^4 \\ K_2 &= (ML)^4/(M)^4(L)^4 \\ K_3 &= (M_4L_i)/(M_4)(L)^i \\ K_4 &= (M_4L_i)(L)^{4-i}/(ML)^4 \end{aligned} \qquad [5]$$

where L is a ligand species such as Ins-P_6. The experimentally determined macroscopic association equilibrium constant for tetramer formation is

$$\begin{aligned} K_T &= [\text{total tetramers}]/[\text{total monomers}]^4 \\ &= K_1[1 + K_3(L)^i]/[1 + K_2^{1/4}(L)]^4. \end{aligned} \qquad [6]$$

From the data of Fig. 2 it may be inferred, at least for the four unliganded chains, that $K_1 \approx K_4$, since $K_T = K_4$ at saturating values of (L). Then (and since $K_1K_3 = K_2K_4$),

$$K_T = K_1 \frac{[1 + K_2(L)^i]}{[1 + K_2^{1/4}(L)]^4}. \qquad [7]$$

Examination of this general expression for cases $i = 1, 2, 3$, and 4, reveals that K_T may be expected to vary significantly with (L) for all cases of stoichiometry. In the least extreme case, $i = 4$, $K_T/K_1 = 1/8$ at an effector concentration equal to $K_2^{-1/4}$. Since even this range of variation is far outside the limits observed, it may be concluded that appreciable binding of Ins-P_6 to the β chains does not occur under conditions of these experiments.

CONCLUDING REMARKS

For a number of years the dominant concept of cooperative interactions in multisubunit proteins, and particularly in hemoglobin, has embodied the assumption that assembly of subunits into quaternary structures in the unliganded state leads to structural and thermodynamic constraints that are reflected in a decreased affinity at the binding sites. The binding affinity then becomes greater (larger negative free energy) with increasing state of ligation as the thermodynamic and structural constraints are released. The various theories of allosteric regulation have been aimed at describing the molecular details whereby these overall processes may be carried out.

Based upon current understanding of protein structure and function, there is no reason in principle why the assembly of protomers into quaternary structures might not lead to an *increased* binding affinity for ligands, although no case of this has, to our knowledge, been reported prior to the present study. Although the β_4 tetramers have not been found to exhibit cooperative binding, they comprise a hemoglobin system (hemoglobin H) in which the energetics of quaternary structure formation and ligation are linked in this "reverse" fashion. It seems likely that more cases of this phenomenon may be revealed as results of detailed thermodynamic studies are carried out for a wider range of systems.

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