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Quaternary enhancement in binding of oxygen by human hemoglobin

(allostery/thermodynamics/proteins/linkage)

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ABSTRACT From an experimental study on the linkage between dimer–tetramer association and oxygen binding in human hemoglobin it is found that triliganded tetramers $\alpha_2\beta_2(O_2)_3$ have an affinity for oxygen that is significantly higher than that of $\alpha^1\beta^1$ dimers (superscripts denote intersubunit contacts). This conclusion is based upon a newly determined series of accurate oxygen binding isotherms, which were analyzed in conjunction with independently determined values of the dimer–tetramer equilibrium constants in the unliganded and fully oxygenated states [Ip, S. H. C. & Ackers, G. K. (1977) *J. Biol. Chem.* 252, 82–87]. The results imply that in the molecule $\alpha_2\beta_2(O_2)_3$ the interactions at the $\alpha^1\beta^2$ intersubunit contacts are propagated to the unliganded heme in a manner that *increases* its affinity for oxygen. This effect contrasts sharply with the well-known reduction in oxygen affinity arising from these same contacts when unliganded dimers are assembled to form unliganded $\alpha_2\beta_2$ tetramers. The magnitude of the enhancement in affinity at the unliganded site in triliganded tetramers (0.81 kcal, 3.39 kJ) is approximately one-fourth as great as the reduction in affinity on each heme site that arises from subunit assembly of the unliganded tetramer. The terms "quaternary constraint" and "quaternary enhancement" are employed to describe these oppositely directed effects of intersubunit interaction upon heme-site affinity. Experimental results also suggest that dimers bind oxygen with a higher affinity than monomeric α and β chains do under the same temperature and buffer conditions (21.5°C, pH 7.40, 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA). Thus quaternary enhancement may be manifested at the $\alpha^1\beta^1$ contacts. Implications of these results for models of the cooperative mechanism are discussed.

Assembly of unliganded α and β chains of human hemoglobin into the $\alpha_2\beta_2$ tetramer results in a quaternary structure that has a greatly reduced affinity for oxygen relative to the isolated chains. Interactions between the assembled subunits are thus propagated to the heme sites in a manner that reduces the affinity by a factor of $\approx 1/250$ for the first oxygen bound. This reduction in affinity is generally believed to be the result of "constraints" (structural and thermodynamic) that are imposed at the heme sites as a result of intersubunit interactions (cf. ref. 1 for a general review). As additional oxygen molecules are bound, these constraints are altered in such a way that the affinities are increased, until at the last step the affinity more nearly approximates that of the isolated chains. X-ray structural analysis has provided a stereochemical rationale for the existence of oxygen-sensitive structural constraints (1) and the various theories of allosteric regulation have been aimed at describing the molecular details whereby their "release" is accomplished (cf. ref. 2 for a general review). The term "quaternary constraint" was coined by Monod *et al.* (3) to denote alterations in conformation arising from subunit assembly, which in their model leads to a decrease in ligand affinity at sites remote from the subunit contacts.

Recently an effect of the opposite kind has been found in chains of β human hemoglobin (hemoglobin H) (4, 5). In that system, the interactions between assembled subunits are propagated to the heme sites in a manner that *enhances* their affinity for oxygen and carbon monoxide. A similar result has been reported for the binding of oxytocin and vasopressin to neurophysin, which occurs preferentially onto dimers rather than monomers (6, 7). In considering such effects further it is useful to define the term "quaternary enhancement" to denote an increase of ligand binding affinity upon assembly of isolated subunits into a quaternary structure. For the opposite effect we shall use the term "quaternary constraint" as considered by Monod *et al.* (3). It should be noted that these terms represent a different concept from that which is commonly referred to as "positive cooperativity" or "negative cooperativity" (or "anticooperativity"). The latter concept refers solely to the direction of changes in binding affinities for successive steps, with increasing affinities denoted as "positive" and decreasing ones as "negative" (or "anti"). The concept of quaternary constraint or quarternary enhancement defines the relationship of such changes in a multisubunit protein to a reference state of the molecular constituents—i.e., the dissociated subunits. Positive cooperativity may arise from a progressive release of quaternary constraints with ligand binding steps, or from progressive appearance of a quaternary enhancement effect, or from a combination of both. Various roles of quaternary enhancement and quarternary constraint effects have been considered previously within the framework of particular allosteric models (cf. ref. 8). It is useful, however, to consider them in a less restrictive, model-independent context, as general thermodynamic properties of macromolecules. As such, they may be determined empirically and without recourse to additional assumptions.

In this paper we report the experimental finding that $\alpha^1\beta^1$ dimers‡ have an affinity for oxygen that is significantly lower than that of triliganded $\alpha_2\beta_2$ tetramers. As a result of the linkage between dimer–tetramer assembly and oxygen binding, the association of two $\alpha^1\beta^1$ dimers (one singly liganded, the other doubly liganded) results in a quaternary structure $\alpha_2\beta_2(O_2)_3$ with enhanced binding affinity at the last site. The interactions between subunits at the $\alpha^1\beta^2$ contacts are thus propagated to the heme sites as a "quaternary enhancement" effect, increasing the affinity at those sites. We also present evidence that quaternary enhancement probably exists in the $\alpha^1\beta^1$ dimers. These dimers are found to have higher affinities for oxygen than the mean value for monomeric α and β chains determined under the same conditions.

The approach used in this work was to study the linkage

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‡ Superscripts denote the known intersubunit contacts ($\alpha^1\beta^1$ or $\alpha^1\beta^2$) for the species considered, whereas subscripts, as usual, denote subunit composition. The normal hemoglobin tetramer $\alpha_2\beta_2$ has two types of contacts between dissimilar subunits, denoted $\alpha^1\beta^1$ and $\alpha^1\beta^2$ (1). Dissociated dimers are of the $\alpha^1\beta^1$ type and are assumed in all analyses to bind oxygen noncooperatively (6, 9, 10, §).

Table 1. Total free energies of binding oxygen by dimers and tetramers*

Data set	Hemoglobin, μM	K_{44}† $\times 10^{-18}$ (4 mol O_2 bound)	ΔG_4, kcal/4 mol O_2 bound	ΔG_2,‡ kcal/2 mol O_2 bound
1§	382, 76.5, 38.2, 5.4	164 ± 4.0	−27.25 ± 0.05	−16.75 ± 0.25
2	86.8, 4.52	138 ± 11	−27.14 ± 0.05	−16.69 ± 0.2
3	70.9, 53.1, 47.6, 9.66	112 ± 8.9	−27.03 ± 0.05	−16.62 ± 0.2
4	43.5, 43.2, 41.6, 4.8	117 ± 9.3	−27.05 ± 0.05	−16.64 ± 0.2

1 kcal = 4.184 kJ.

* Conditions: 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA, pH 7.40, 21.5°C.

† Calculated from the medians of the binding curves and the dimer–tetramer equilibrium constants $^0K_2$, $^4K_2$ according to procedures described in ref. 12.

‡ $\Delta G_2 = \frac{1}{2}(\Delta G_4 + {}^0\Delta G_2 - {}^4\Delta G_2)$; ${}^0\Delta G_2 = -RT \ln {}^0K_2$, in which $^0K_2 = 3.57 \times 10^{10}\ M^{-1}$ and ${}^4\Delta G_2 = -RT \ln {}^4K_2$, in which $^4K_2 = 8.65 \times 10^5\ M^{-1}$ (7).

§ Detailed analysis of this data set is described in ref. 11.

between association of $\alpha^1\beta^1$ dimers into $\alpha_2\beta_2$ tetramers and the binding of oxygen by these species (11–13). This information has been combined with results of recent studies on the assembly of β_4 tetramers as a function of oxygenation state (4) and with accurate measurements of oxygen binding by isolated α and β chains,‡§ all determined under identical conditions.

In a previous study (12) we found evidence that suggested the intrinsic free energy of formation for triliganded tetramers may be more positive than the corresponding energy for fully oxygenated tetramers. Such a difference in free energies of formation would require the affinity of triliganded tetramers to be higher than that of singly liganded dimers, as a result of the linkage between oxygen binding and dimer–tetramer association (11). The recent demonstration of quaternary enhancement in the assembly of β chains (4, 5), plus the results of an extensive series of oxygenation studies on isolated chains§ indicating their affinities to be substantially lower than the affinity of triliganded tetramers, provided additional incentive to explore these effects further.

METHODS

The major component of human hemoglobin (A_0, free of organic phosphates) was prepared from three separate donors by the method of Williams and Tsay (14). All experiments were conducted at 21.5°C in 0.1 M Tris·HCl buffer/0.1 M NaCl/1 mM Na_2EDTA at pH 7.40.

Oxygen binding curves were determined as a function of hemoglobin concentration by using the automatic technique of Imai *et al.* (9) as previously applied to this linkage problem (12). The data were analyzed in conjunction with independently determined values of the dimer–tetramer association constants, $^0K_2$ and $^4K_2$ pertaining to unliganded and fully oxygenated hemoglobin, respectively. Values of these constants were calculated from the van't Hoff enthalpies and entropies derived from temperature dependence studies carried out between 5°C and 37°C (13). The values estimated in this way lie within the error limits of the values determined at 21.5°C that were used in our earlier studies (12).

Numerical analysis of the oxygenation curves was carried out according to procedures that have been described in extensive detail elsewhere (refs. 10, 12; see ref. 12 for a critical discussion on the validity of equilibrium constants estimated by these procedures; ref. 10 describes the effects of errors on the numerical fitting problem). The criteria used for evaluation of data included: uniqueness of least-squares minima, variance of fit, randomness in distribution of residuals, correlation properties of fitted parameters as compared with numerical simulations of error-perturbed data over the same range of conditions, and internal self-consistency of results from fitting different combinations of the linkage parameters.

In this study additional simulations and analyses were devised to evaluate possible effects upon the final results of inaccuracies in the estimation of end-points for the oxygenation curves. In principle, errors in these quantities could have serious effects on the estimation of equilibrium constants because they determine the total span of the normalized $\bar{Y}$ function and thus indirectly affect the shape of the oxygenation curves, particularly in the regions of high and low fractional saturation. Usually at least one of the end-points is estimated by extrapolation (12), and thus some error is to be expected simply as a result of the extrapolation procedures. In this study, the effects of perturbations on the estimated end-points of the experimentally determined binding curves were explored systematically to evaluate their effects upon resolvability of the linkage parameters. Increments corresponding to errors in determining either the 0% or 100% saturation end-points were added or subtracted in all possible combinations, and the resulting curves were analyzed. Results of these analyses indicated the perturbations to have no substantial effect upon estimated values of the linkage parameters unless the perturbations were larger than 0.006 (in $\bar{Y}$ units). The maximum errors of end-point determination in practice were estimated to be considerably smaller and only a slight broadening of the confidence limits was found to arise from them.

Additional studies were also carried out to explore the effects upon the linkage analysis of varying $^0K_2$ and $^4K_2$ within their experimentally determined ranges of error. These studies are described in *Results*.

The protein concentrations employed in the new data sets are given in Table 1. No oxygenation curves were measured at concentrations below 1 μM heme because the previous studies (10, 12) had shown the data at these low protein concentrations to be of decidedly reduced value for reliable evaluation of the linkage parameters. Even at the higher concentrations employed in this study a pronounced dependency of oxygenation curves upon protein concentration was observed, reflecting the contribution from high-affinity dimers to the overall binding isotherms. This is illustrated in Fig. 1.

RESULTS

The data sets from the three separate donors were found to be of sufficiently high quality to permit complete resolution of the linkage parameters when combined with the independently determined equilibrium constants for dimer–tetramer associ-

§ F. C. Mills, G. K. Ackers, H. T. Gand, and S. J. Gill, *J. Biol. Chem.*, in press.

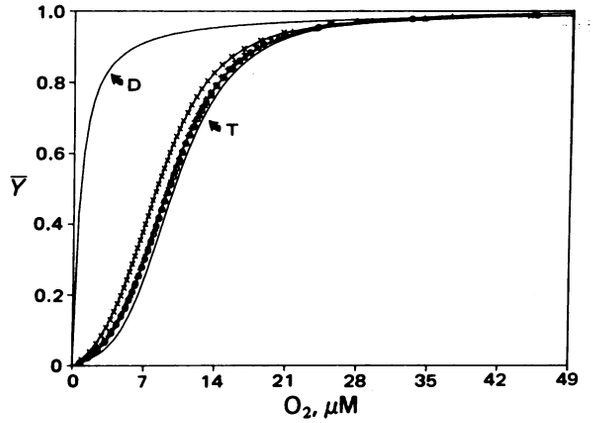

FIG. 1. Oxygenation data for human hemoglobin at pH 7.40, 21.5°C (0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA). Data set 3. Points are plotted for curves obtained at protein concentrations of 70.9 (○), 53.1 (△), 47.6 (+), and 9.66 (×) μM heme. Solid curves are drawn from least squares fit to the data by procedures (5, 6) that take into account the presence of dimers and the dimer–tetramer equilibria as well as the oxygen binding by dimers and tetramers. Solid curves at extreme left (D) and right (T) are the calculated isotherms for dimers and tetramers, respectively.

ation of unliganded and fully oxygenated species. The analyses were carried out in two stages:

(*i*) Determination of equilibrium constants and free energies for totally oxygenating tetramers and dimers

The equilibrium constant K_{44} for binding four oxygens by the tetramer was calculated from the median of each binding curve and values of ${}^0K_2$ and ${}^4K_2$ (10). Average values of K_{44} for each data set are listed in Table 1, along with the corresponding free energies ΔG_4 for fully oxygenating the tetramers. Values of ΔG_2, the free energy for binding two oxygens onto dimers, are also given in Table 1. Excellent agreement was found between the free energies calculated from the three new data sets from different hemoglobin preparations (Table 1).

The agreement between results of the two groups of experiments (set 1 and sets 2, 3, 4), carried out several years apart, is also quite close. The median affinities found in the new data sets were all slightly reduced relative to the affinities of curves at corresponding concentrations in the previous study (data set 1). This small discrepancy was attributed to the pH of the buffer in the first experiments being 0.03 to 0.06 pH units higher than 7.40, as a result of poorer temperature control while the buffers used in set 1 were made.

Table 3. Free energy changes (kcal/mol) corresponding to the last step of oxygen binding by human hemoglobin tetramers and dimers

Data set	$\delta\Delta G'_{34}$*	$\Delta G'_{44}$†	$\Delta G'_{22}$‡
1	0.27 ± 0.22	−8.65 ± 0.12	−8.38 ± 0.22
2	0.77 ± 0.31	−9.10 ± 0.27	−8.35 ± 0.18
3	0.86 ± 0.57	−9.18 ± 0.54	−8.31 ± 0.18
4	1.33 ± 0.83	−9.65 ± 0.79	−8.32 ± 0.18
Mean	0.81 ± 0.27	−9.15 ± 0.25	−8.34 ± 0.09

* $\delta\Delta G'_{34} = {}^4\Delta G'_2 - {}^3\Delta G'_2$. All values have been corrected for statistical factors.

† Free energies for the last step of oxygen binding to tetramers, representing the affinity of triply liganded hemoglobin: $\Delta G'_{44} = \Delta G'_{22} - \delta\Delta G'_{34}$.

‡ Free energies for the last step of oxygen binding by dimers, assuming noncooperative dimers and correcting for statistical factors.

(*ii*) Analysis for the remaining linkage parameters

The newly determined sets of oxygen binding curves were analyzed for the remaining linkage parameters in a manner similar to that described previously for data set 1 (12). Results of the analyses are given in Tables 2 and 3 as intrinsic free energies—i.e., corrected for statistical factors.

Values of the intrinsic free energies of formation for tetramers from dimers in various stages of ligation are given in Table 2. Resolution into energy differences corresponding to four of the five ligation states was obtained with good accuracy. The problems associated with resolving energies of formation for doubly liganded tetramers have been discussed elsewhere (11, 12). It can be seen from Table 3 that the results of analyses on the new data sets define a sequence of changes in energetic state accompanying oxygenation that are highly consistent among samples of hemoglobin derived from different donors and also with the results obtained earlier with a single preparation (set 1). The four data sets support the conclusion drawn previously (12) that there are a minimum of three separate energy states for the hemoglobin tetramers as a function of oxygenation [(*i*) unliganded, (*ii*) singly liganded, (*iii*) triply and fully oxygenated]. The new results provide additional resolution between the third and fourth steps. Because the differences between ${}^3\Delta G_2$ and ${}^4\Delta G_2$ are consistently larger than the errors associated with the determined values, these results define a minimum of four separate energy states, corresponding to the degrees of ligation shown in Table 2.

A listing is given in Table 3 of the differences $\delta\Delta G'_{34}$ found between free energies of tetramer formation for the species participating in the last binding step. The fact that the values

Table 2. Free energies (kcal/mol) of dimer–tetramer association for human hemoglobin in various states of oxygenation*

Data set	${}^0\Delta G'_2$	${}^1\Delta G'_2$	${}^3\Delta G'_2$	${}^4\Delta G'_2$
1†	−14.34 ± 0.14	−11.46 ± 0.2	−7.78 ± 0.12	−8.05 ± 0.10
2	−14.23 ± 0.05‡	−11.26 ± 0.16	−7.24 ± 0.27	−8.00 ± 0.04‡
3	−14.23 ± 0.05‡	−11.28 ± 0.23	−7.14 ± 0.53	−8.00 ± 0.04‡
4	−14.23 ± 0.05‡	−11.34 ± 0.16	−6.68 ± 0.79	−8.00 ± 0.04‡
Mean	−14.26	−11.34	−7.21	−8.01

* The symbol ${}^1\Delta G'_2$ represents the free energy of formation for 1 mol of tetramers with 1 mol of oxygen molecules bound. The errors represent confidence limits on the estimated parameters at 1 SD.

† Values derived from the study reported in ref. 12.

‡ Calculated from Van't Hoff enthalpies and entropies determined independently under conditions identical to those of this study: 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA, pH 7.40, 21.5°C.

Table 4. Comparison between affinities of isolated α and β chains and $\alpha\beta$ dimers*

ΔG_α†	ΔG_{β_4}†	ΔG_β‡	$\Delta G_\alpha + \Delta G_\beta$	ΔG_2§	$\Delta G_\alpha + \Delta G_{\beta_4}$
−8.11 ± 0.08	−8.56 ± 0.12	−7.72 ± 0.3	−15.83 ± 0.36	−16.65 ± 0.3	−16.67 ± 0.11

* Free energies are in kcal per mol of O_2 bound. The reference state is 1 mol of O_2 (aq) per liter, 21.5°C, pH 7.4, 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA.

† Ref. 14

‡ Corrected for linkage energy as determined in ref. 5.

§ ΔG_2 determined in this study: $\Delta G_2 = \Delta G_4 + {}^0\Delta G_2 - {}^4\Delta G_2$.

of $\delta\Delta G'_{34}$ are consistently positive indicates the free energy of tetramer formation to be more positive in going from the fully oxygenated to the triply liganded molecule (i.e., dimers are less "tightly" associated). With further deoxygenation the sign of this effect is reversed, as shown in Table 2. Thus the intersubunit constraints are seen to operate in two directions, depending upon oxygenation state.

Another manifestation of the same phenomenon can be seen by comparing the free energies ΔG_{44} for oxygen binding to triply liganded tetramers with the energy $\Delta G'_{22}$ of reaction with singly liganded dimers (the latter being taken as the mean intrinsic binding energy per heme for dimers as a whole, $\Delta G_2/2$). As shown in Table 3, the binding affinity for the tetramers is greater by an average value of 0.81 kcal per mol of O_2. Thus assembly of the appropriate combination of dimers to form the triply liganded tetramer leads to quaternary enhancement for binding of the last oxygen. The magnitude of the enhancement (0.81 ± 0.3 kcal per mol of O_2) is found to be approximately the same as that recently reported (4) in the assembly of β_4 tetramers (0.84 ± 0.3 kcal per mol of O_2).

Effects of changing ${}^0K_2$ and ${}^4K_2$

It was found that values of the variance and of the estimated linkage parameters were fairly insensitive to raising ${}^0K_2$ to the limit allowed by the confidence intervals on the van't Hoff enthalpy and entropy for this quantity, and the value of $\delta\Delta G_{34}$ remained positive. On the other hand, the fitting problem was found to be extremely sensitive to variations in ${}^4K_2$. Increasing the total linkage by lowering ${}^4K_2$ from 8.65×10^5 M^{-1} to 6×10^5 M^{-1} caused the program to estimate impossibly high values for some of the linkage parameters, and the variance of the fit became twice as large. The oxygenation curves were found to define narrow limits for ${}^4K_2$; raising its value from 8.65×10^5 M^{-1} to 1×10^6 M^{-1} was found to increase the variance 4-fold. Increasing ${}^0K_2$ to 1.973×10^{11} M^{-1} and decreasing ${}^4K_2$ to a value of 4×10^5 M^{-1} caused the program to estimate a negative value for one of the binding constants, and raised the variance by more than an order of magnitude. From results of these studies it was concluded that even though increasing the ratio of ${}^0K_2$ to ${}^4K_2$ would diminish the positive value of $\delta\Delta G_{34}$, the fits to the data obtained by this procedure are greatly inferior to those obtained by using the most probable values as determined independently for these constants.

Quaternary enhancement in the assembly of $\alpha\beta$ dimers

A quaternary enhancement effect is also suggested by the comparison of values for ΔG_4 and ΔG_2 with the energies for binding to the isolated α and β chains. Under conditions identical to those of this study these latter energies have recently been determined[§] as: −8.11 ± 0.08 kcal per mol of O_2 for α chains (the same value for monomers or dimers) and −8.56 ± 0.12 kcal per mol of O_2 for β chains (in the form of β_4 tetramers). The sum of free energies for binding one oxygen onto an isolated α chain and one oxygen onto a β_4 tetramer thus equals −16.67 kcal per 2 mol of O_2. However, the oxygenation of β chains is linked to aggregation, so that the binding energy for a dissociated β chain would be lower by −0.84 kcal per mol of O_2 (4). The corresponding sum would therefore be −15.83 kcal per 2 mol of O_2. The mean value for ΔG_2 from data sets 2, 3, and 4 (Table 1) is −16.65 kcal per 2 mol of O_2. These values are summarized in Table 4 along with their associated statistical uncertainties. The free energy ΔG_2 for totally oxygenating dimers is seen to be nearly identical to the sum of energies per heme for oxygenating an isolated α chain (α or α_2) plus a β_4 chain, but the energy is significantly higher than the corresponding sum for the β chain in its monomeric form. The difference between ΔG_2 and $\Delta G_\alpha + \Delta G_\beta$ is found to be significantly larger than the errors defined by the confidence limits at one standard deviation (Table 4). Thus these results strongly suggest an enhancement of binding affinity when monomeric chains are converted into $\alpha\beta$ dimers.

DISCUSSION

Because the significant thermodynamic effects upon which cooperative mechanisms are based arise as small differences between larger energetic quantities, it is of crucial importance to obtain an extensive and self-consistent set of experimental information under constant conditions. In the present study meticulous effort has been directed toward this goal. Much of the documentation for reliability of the procedures employed has been presented in extensive detail elsewhere (10,·12) and has not been repeated here. Based on these studies and the additional controls described here, the energetic values derived are accurate to within the specified error limits and the corresponding differences found are significant.

From the results of this study we conclude that the free energy for binding oxygen to triply liganded hemoglobin tetramers has a significantly larger negative value (higher affinity) than the mean free energy for dimers or for isolated chains. Thus the hemoglobin system exhibits quaternary enhancement for oxygen binding in addition to the quaternary constraint effect that has long been known. The magnitude of the enhancement in affinity at the unliganded site in triliganded tetramers (0.8 kcal/mol) is approximately one-fourth as great as the reduction in affinity (2.89 kcal/mol) at each site that arises from assembly of unliganded tetramers (i.e., ${}^1\Delta G_2 - {}^0\Delta G_2$). A correspondingly significant structural difference may therefore be expected between the unliganded chains in $\alpha_2\beta_2(O_2)_3$ and those in the fully unliganded tetramer $\alpha_2\beta_2$. We do not know of any experimental measurements presently capable of resolving these structural differences. The kinetic basis of the observed thermodynamic differences would also be of interest, but would require data of accuracy comparable to that described in these studies. Such data does not appear to exist within the literature. In addition to the results presented here, recent studies (to be published elsewhere) show that quaternary enhancement effects are present in human hemoglobin at all temperatures between 10°C and 37°C. Those results and the finding here of quaternary enhancement in hemoglobins from a variety of separate donors provide additional evidence that the effect is real. It would be of interest for future studies to determine whether it is modulated by the presence of organic phosphates or by the binding of protons.

The existence of oppositely directed thermodynamic effects within the hemoglobin tetramer has important implications for theories of the relationship between mechanism and structure. For example, the model of Szabo and Karplus (15) applied to the data of Roughton and Lyster (16) predicts a higher intrinsic affinity for hemoglobin chains within the tetrameric molecule as compared to the literature values for affinities of the isolated α and β subunits. Up to this point there have been no model-independent results that demonstrate unequivocally that effects of this kind actually occur in normal human hemoglobin—i.e., that assembly of the subunits can produce quaternary structures with enhanced binding affinity. In considering the relationship of this finding to theories of hemoglobin, it is of interest to note two general mechanistic interpretations.

(*i*) It may be assumed that all modulating effects of quaternary structure upon heme-site ligand affinity are sensitive to oxygen binding, but that they change in two directions during the course of oxygenation. Starting with the deoxygenated tetramer, the quaternary constraints are progressively released during successive oxygen binding steps up to a certain stage (e.g., the second step), after which a quaternary enhancement effect is created, giving rise to increased affinity at the heme sites.

(*ii*) Alternatively, it may be assumed that structural features that give rise to the two classes of effects coexist within the same tetramer, and that only the quaternary constraints change upon oxygenation. Thus assembly of subunits into tetramers might result in quaternary enhancement of nearly constant magnitude regardless of oxygenation state. In the deoxygenated molecule they would be masked by the larger quaternary constraints that serve to reduce affinity. As the constraints are released progressively upon oxygenation, a point is reached at which their effect becomes smaller than the remaining enhancement effect.

The above schemes serve to illustrate the two major categories of possibilities, although many additional combinations and variations can be envisioned. Current theories of hemoglobin mechanism would require the least alteration if scheme (*ii*) were correct because the oxygen-sensitive properties of the tetramer could still be explained entirely on the basis of quaternary constraints. Very drastic alterations, however, would be required to accommodate scheme (*i*), because the creation of structural features upon oxygenation that increase oxygen affinity would be required. In any case, it is no longer possible to consider the effects of intersubunit interactions upon oxygen affinity solely in terms of a reduction in heme site affinity. The results presented here, and the recent findings of quaternary enhancement in hemoglobin H (5, 6), point to the need for new information regarding the structural basis of these effects.

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