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COOPERATIVE INTERACTIONS OF HEMOGLOBIN

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CONTENTS

INTRODUCTION 209
FORMULATIONS OF THE PROBLEM OF COOPERATIVE LIGAND BINDING 210
Early Models 210
The Adair Scheme 211
The Sequential Models 211
The Two-State Model 213
THE MINIMUM UNIT OF COOPERATIVITY 216
DEDUCTIONS FROM STRUCTURAL STUDIES 219
DETECTION OF THE $T \leftrightarrow R$ TRANSITION 221
EVIDENCE FOR NONEQUIVALENCE OF THE α AND β CHAINS 223
Basic Observations 223
Consequences of Chain Nonequivalence 225
GENERAL CONCLUSIONS AND CURRENT ISSUES 226

INTRODUCTION

Hemoglobin has been studied actively since the 19th century and reviewed on numerous occasions, including an article in this series in 1970 (1). Progress in understanding the molecule continues at a rapid pace and a number of important developments have occurred in recent years which warrant review at this time, including: (*a*) a more detailed understanding of the molecular structure from X-ray crystallographic studies, with direct implications for the mechanism of cooperative binding of oxygen by hemoglobin; (*b*) a recognition of significant differences in the functional properties of the individual α and β chains of hemoglobin; (*c*) the demonstration that isolated $\alpha\beta$ dimers, products of dissociation of the tetrameric $\alpha_2\beta_2$ hemoglobin molecule, are actually devoid of cooperativity and not highly cooperative as believed at the time of the last review; and (*d*) advances in the application of quantitative models, based on new physical-chemical studies, to describe the various features of hemoglobin reactions. The restrictions in length imposed on a review in these volumes prohibit a full exposition of all relevant topics. Therefore, this review focuses on the central and long-standing

question that dominates hemoglobin research: What is the structural basis for "heme-heme" interactions, i.e. the cooperative binding of oxygen and other ligands by hemoglobin? Following a discussion of the major conceptual formulations of cooperative interactions, those recent studies that bear on elucidating the mechanism of cooperativity in mammalian hemoglobin will be emphasized.

More complete descriptions of earlier work and background information can be found in existing reviews (1–9), including an excellent historical treatment that has recently appeared (10). An important role has been played by mutants of human hemoglobin in elaborating structure-function relationships, and selected examples are drawn from this source as they relate to individual topics. However, no attempt is made to summarize the vast amount of work that has been done on mutant forms, much of which has been amply reviewed (1, 6, 11, 12). The special problems associated with sickle cell hemoglobin were summarized in June 1974 at the first National Symposium on Sickle Cell disease, and the proceedings of this meeting are to be published.

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FORMULATIONS OF THE PROBLEM OF COOPERATIVE LIGAND BINDING

Cooperativity is manifested most directly by the sigmoid curve for the fractional saturation of hemoglobin as a function of the partial pressure of oxygen. In contrast to the sigmoidal behavior, noncooperative binding is reflected by a hyperbolic curve. It is this physiologically important feature, the sigmoid curve, that investigators have for so long attempted to understand; other important properties, such as the Bohr effect and organic phosphate effects, can be viewed as adjustments of the basic phenomenon.

Early Models

Early explanations of cooperative oxygen binding by hemoglobin anticipated the mechanistic alternatives that still confront researchers. The first important mechanism was presented by Hill (13), who postulated that cooperativity arose by a concerted reaction of n binding sites on hemoglobin and could be described by the equation

$$\frac{Y}{1-Y} = Kp^n \qquad 1.$$

where Y is the fractional saturation, p is the partial pressure of oxygen, and K is an equilibrium constant. Modern data can generally be fit with this equation for values of $Y = 0.1$–0.9 to yield a Hill constant, $n \approx 3$. However, the equation is now only a convenient index of cooperativity; the implications in terms of a mechanism of concerted reaction at n sites cannot be correct, since (*a*) the number of oxygen binding sites is four (14); (*b*) the value of n approaches unity as Y approaches 0 or 1 (15); and (*c*) the kinetics of ligand binding are not consistent with a high order reaction (16).

Another early model of cooperativity was proposed by Douglas, Haldane & Haldane (17) based on a higher degree of aggregation for deoxyhemoglobin than

oxyhemoglobin. The finding of Adair (14) of molecular weights (~65,000) corresponding to four heme-containing units (1 iron/16,000) for both oxy- and deoxyhemoglobin also disqualified this theory. However, the variable aggregation idea has found application for lamprey hemoglobin (18–20) and anticipated the relaxed and constrained states in the theory of Monod et al (21), in which differences in aggregation equilibrium constants are present, although not necessarily revealed in ligand-dependent dissociation, when ligand binding occurs at protein concentrations well above the subunit dissociation constants (see below).

The Adair Scheme

In addition to disqualifying the theories of Hill & Douglas et al, the work of Adair laid the foundation for the modern formulation of the problem of cooperativity by describing a phenomenological equation (22). Since four oxygen molecules are bound per heme, the oxygen binding equilibria can be described by

$$Y = \frac{K_1p+2K_1K_2p^2+3K_1K_2K_3p^3+4K_1K_2K_3K_4p^4}{4(1+K_1p+K_1K_2p^2+K_1K_2K_3p^3+K_1K_2K_3K_4p^4)} \qquad 2.$$

where the individual equilibrium constants K_i are related to the reaction

$$Hb(O_2)_{i-1}+O_2 \rightleftharpoons Hb(O_2)_i \qquad 3.$$

for $i = 1$–4 and p is the partial pressure of oxygen. This formulation merely describes the system in the broadest terms. The individual binding constants may be determined and efforts along these lines, principally by Roughton and his co-workers (23), revealed that cooperative binding of oxygen is expressed by a value of K_4 some 300 times greater than K_1, when corrected for statistical factors. However, in itself the Adair formation provides no indication of a structural mechanism.

The Sequential Models

The first effort to provide a structural mechanism was a model proposed by Pauling in 1935 (24) which is still relevant with today's knowledge. He argued that the four Adair constants could be expressed in terms of a fundamental oxygen binding equilibrium constant, K', and an interaction constant, α, which describes the stabilization resulting from adjacent oxygen-containing heme units. For a tetrahedral arrangement of hemes, cooperativity arises because the binding of the first oxygen molecule is accompanied by no interheme stabilization, with interactions increasing with each oxygen bound until binding of the fourth oxygen molecule, which is accompanied by three stabilizing interactions. Therefore, the four Adair constants can be expressed as the product of the appropriate statistical factor, the fundamental constant, K', and the interaction constants

$$\begin{aligned} K_1 &= 4K' \\ K_2 &= \tfrac{3}{2}K'\alpha \\ K_3 &= \tfrac{2}{3}K'\alpha^2 \\ K_4 &= \tfrac{1}{4}K'\alpha^3 \end{aligned} \qquad 4.$$

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In this formulation the value of K_4 is greatly enhanced over K' by the added stabilization of heme units. A second related formulation could be derived in which the interactions between oxygen-containing heme units lead to a destabilization. The greatest destabilization occurs with the binding of the first oxygen molecule; the smallest destabilization accompanies the binding of the last oxygen molecule, and cooperativity occurs. In this case, the four Adair constants would be given by the series listed above, each divided by α^3. Thus, mechanisms in which structural factors influence binding energy can be formulated in two alternative ways, which may be called "structural promotion" and "structural constraint." In the case of structural promotion, oxygen binding is facilitated by stronger structural interactions with successive binding steps to give cooperativity. In the case of structural constraint, oxygen is bound while overcoming structural stabilization that diminishes with successive binding steps.

The original formulation of Pauling assumed structural promotion, and this idea was carried through to the modern version of the Pauling model by Koshland, Nemethy & Filmer (25). In contrast, the opposite premise, structural constraint, is an essential feature of the model of Monod et al (21). We now know the structural constraint alternative prevails for hemoglobin. Wyman (2) was the first to conclude that Pauling was incorrect in using structural promotion, on the basis of increased oxygen affinity in the presence of urea, a structure-disrupting agent. More convincing is the finding that α and β chains of hemoglobin have an affinity for oxygen approximately 30 times higher than intact hemoglobin (26). Moreover, the binding constant for chains is in close agreement with the value for the fourth Adair constant. Therefore, the "fundamental" binding properties of hemoglobin sites are exhibited only in the last step. The early steps are reduced in affinity by structural constraint.

Koshland et al (25) extended the Pauling model to other geometric arrangements and other categories of stabilizing interactions. Two distinct conformations, A and B, were assumed with an isomerization equilibrium $K_t = (B)/(A)$. Ligand (S) binding to A induces conformation B, so that the fundamental affinity constant K' of Pauling (24) is given by $K' = K_t K_s$, where $K_s = (BS)/(B)(S)$. The most important addition to the Pauling model was the inclusion of a factor related to stabilizing interactions between mixed pairs. In addition to the relative stabilization of liganded protein over unliganded, K_{BB} (equivalent to the α of Pauling), Koshland et al defined a parameter K_{AB} which gives the relative stabilization arising from type A subunits in contact with type B subunits. This term is very critical to predictions by the model since cooperativity is highly sensitive to its value (27). In the extreme, as K_{AB} goes to zero, the Pauling-Koshland formulation becomes equivalent to the Hill equation. With the terms defined by Koshland et al (25), the Adair constants take the form

$$\begin{aligned} K_1 &= 4(K_s K_t)K_{AB}^3 \\ K_2 &= \tfrac{3}{2}(K_s K_t)K_{BB}K_{AB} \\ K_3 &= \tfrac{2}{3}(K_s K_t)K_{BB}^2 K_{AB}^{1} \\ K_4 &= \tfrac{1}{4}(K_s K_t)K_{BB}^3 K_{AB}^{-3} \end{aligned} \qquad 5.$$

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Since Koshland et al followed the examples of Pauling and continued to define the parameters for hemoglobin in terms of structural promotion, their stabilization constants must be inverted to apply the values to hemoglobin in a physically meaningful way.

The Two-State Model

In 1965 a new formulation of cooperativity was proposed by Monod, Wyman & Changeux (21) which has had a powerful impact on hemoglobin research. The model departed from the earlier sequential ideas and proposed that cooperativity in ligand binding for hemoglobin (or any cooperative proteins) might arise from the existence of just two conformational states. Reasoning from the already identified distinct crystal forms for deoxyhemoglobin and oxyhemoglobin (28), Monod et al noted that the existence of these two conformations was sufficient to generate cooperativity, so long as (*a*) the two states were freely in equilibrium and differed in affinity for ligand, and (*b*) the system was governed by structural constraint. Because of the equilibrium between states, structural constraint is required to maintain the protein in the unliganded conformation in the absence of ligand. To reflect this condition Monod et al designated the predominant state for unliganded proteins as T for tense to emphasize structural constraint and the predominant state for liganded proteins as R for relaxed to emphasize the release of constraint during ligand binding (which facilitates binding in the later states of saturation and leads to cooperativity). Since the structural constraint of the T state will be reflected in interactions between subunits, it was designated "quaternary constraint."

The cooperative binding of oxygen by hemoglobin at pH 7.0 in phosphate was described by an intrinsic equilibrium between the T and R states (defined by $L = [T]/[R]$) where $L = 9054$ (21), with the added assumption that the intrinsic affinity of the T state for oxygen is 0.014 times the intrinsic affinity of the R state ($c = K_R/K_T = 0.014$, where K_R and K_T are dissociation constants for the R and T states, respectively). Descriptively, the model indicates that hemoglobin in the absence of ligand is a mixture of molecules in the T and R states, with T predominating about 10,000 to 1. As oxygen is added both the R and T states are populated, but the binding to the R state is more favorable and the $T \rightleftharpoons R$ equilibrium swings toward the R state. The two states are equally populated when the number of ligand molecules bound is equal to $-\log L/\log c$, or about 2 with the parameters of Monod et al (21). When three molecules of ligand are bound, the R state will predominate and the fourth molecule will be bound with an affinity close to K_R. When the population is saturated the R state will predominate, since $L_4 = (T_4)/(R_4)$, which is equivalent to $L_4 = Lc^4 = 4 \times 10^{-4}$, where the subscript indicates moles of ligand bound. As in the case of the sequential models, the two-state model specifies the Adair constants in terms of structural parameters

$$\begin{aligned} K_1 &= 4(1+Lc)/K_R(1+L) \\ K_2 &= \tfrac{3}{2}(1+Lc^2)/K_R(1+Lc) \\ K_3 &= \tfrac{2}{3}(1+Lc^3)/K_R(1+Lc^2) \\ K_4 &= \tfrac{1}{4}(1+Lc^4)/K_R(1+Lc^3) \end{aligned} \qquad 6.$$

and ligand binding can be described by using the Adair equation with the Adair constants defined in this way. However, the two-state formulation leads directly to a simple equation for Y

$$Y = \frac{\alpha(1+\alpha)^3 + Lc(1+c\alpha)^3}{(1+\alpha)^4 + L(1+c\alpha)^4} \quad 7.$$

where α is the ligand concentration normalized to the dissociation constant of the R state $[\alpha = (X)/K_R]$.

The nomenclature and physical formulation of the two-state model have been widely adopted by hemoglobin researchers in part because of their convenience and in part because the general formulation is consistent with the major observations on hemoglobin, at least to a level of first approximation. These developments arise from certain extensions of the two-state model to the particular properties of hemoglobin. Because of the equilibrium between two states, cooperativity, expressed by the Hill constant n, will be a bell-shaped function of L on a logarithmic scale, as first pointed out by Rubin & Changeux (29). At very low values of L, the R state is present in the absence of ligands, and the $T \rightarrow R$ transition, essential for cooperativity, cannot occur. At very high values of L, the T state is so overstabilized that even the addition of ligand does not provide sufficient energy to favor the R state, and again the $T \rightarrow R$ transition cannot occur. Thus only at optimal values of L will cooperativity occur, with the maximum cooperativity at $L = c^{-i/2}$ where i is the number of binding sites. The bell curve provides a convenient explanation of the Bohr effect absent in the earlier models. The Bohr effect (alkaline) refers to the increase in affinity with pH (from 7–9) with little or no change in cooperativity. In terms of the two-state model, a decrease in L values in the range corresponding to the top of the bell curve would give just such behavior. However, in order to relate L values and experimental observations, one additional extension was required to fix the value of L from oxygen binding data.

As noted by Edelstein (30), since the isolated chains share many of the physical properties ascribed to the R state (31), the binding of oxygen to hemoglobin can be represented by the reaction

$$T + 4(O_2) \rightleftharpoons R(O_2)_4 \quad 8.$$

with an apparent overall binding constant that can be expressed as $p_{1/2}$. Similarly the reaction of chains may be represented by

$$R + 4(O_2) \rightleftharpoons R(O_2)_4 \quad 9.$$

where the apparent binding constant can be expressed by $(p_{1/2})_{\text{chains}}$ and is equivalent to K_R. Solving both equations for L yields

$$L = \alpha_{1/2}^4 \quad 10.$$

where α is $p_{1/2}/K_R$. From this relationship the L value for any oxygen binding curve can be determined where $1 \ll L \ll c^{-4}$. For pH 7 the value of L is deduced to be at least 3×10^5 (30), considerably higher than the original estimate

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of Monod et al (21); in addition the correct value of c is probably lower than the original estimate (30). Thus the numbers of molecules in the R and T states become equal only when three molecules of ligand are bound. When various points are located on the bell curve (see Figure 1*A*), the values of L for pH 7–9 do indeed fall in the region of the top of the bell curve, accounting for the relative invariance of cooperativity in the Bohr effect. Moreover, the behavior of certain affinity mutants can be explained by their positions on the bell curve. The high affinity mutant, hemoglobin Chesapeake (32), and the low affinity mutant, hemoglobin Kansas (33), both of which show low cooperativity, can be explained by their positions on the bell curve. Hemoglobin Chesapeake lies on the left side and is weakly cooperative due to a lack of quaternary constraint. In contrast, hemoglobin Kansas lies on the right side of the bell curve and is only weakly cooperative because it is overconstrained. The decreases in affinity caused by binding of organic phosphates can also be explained by their preferential binding to the T state, which increases L and causes a movement to the right along the bell curve. A further consequence of the two-state formulation is that the value of Y at which the Hill n is a maximum varies between 0.25 and 0.75 in the range of the bell curve (see Figure 1*B*). Because they lack the "buffering" of cooperativity in the midrange of L values (30) the sequential models (25) are less successful in describing the properties of hemoglobin under a wide range of conditions without arbitrary adjustments of parameters.

While the bell curve at a constant value of c is successful for explaining the general form of hemoglobin behavior under many conditions, a precise descrip-

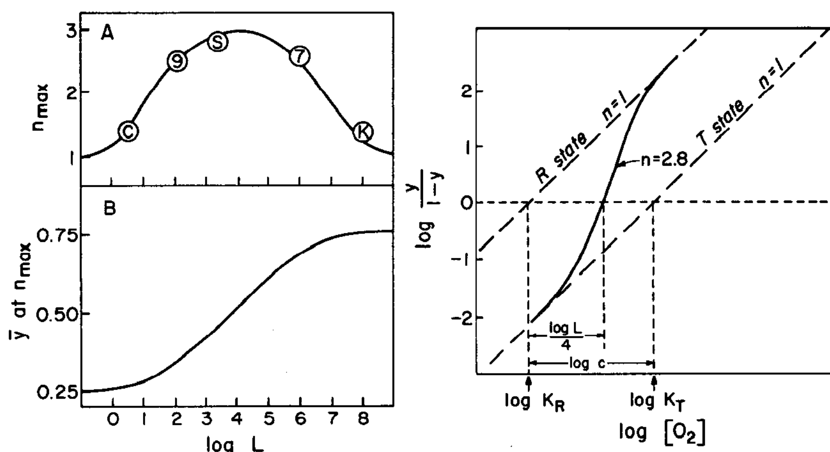

Figure 1 (*left*) (*A*) Bell curve of n_{max} vs log L according to (30). (*B*) Variation in Y at n_{max} vs log L according to (167). Symbols refer to values of n_{max} and log L for hemoglobin Chesapeake (C), hemoglobin Kansas (K), normal hemoglobin at pH 7 (7) and at pH 9 (9), and hemoglobin at pH 7 stripped of organic phosphates (S).

Figure 2 (*right*) Relationship of the Hill plot to parameters of the two-state model.

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tion may require slight changes in c with different conditions. Quaternary constraint leads to stabilization of the T state ($L > 1$) and diminished binding ($c < 1$). The two parameters may be linked in some way so that decreases in L are accompanied by increases in c and vice versa, as some recent data would tend to indicate (34–37). A mechanism for linkage of L and c has been proposed by Szabo & Karplus (38). The distinct effects of L and c can be visualized by considering a Hill plot (Figure 2). The behavior at the extremes relates to the T state at low levels of saturation and the R state at high levels of saturation. If the linear portions at the extremes (with $n = 1$) are extrapolated to a horizontal line at $\log [Y/(1-Y)] = 0$, the intercepts define $\log K_T$ and $\log K_R$; the distance between the intercepts defines $\log c$ (39). The distance from $\log K_R$ to $\log p_{1/2}$ (observed for the experiment) defines $\frac{1}{4} \log L$. Thus c can be estimated directly along with L if data covering a wide range of Y values are available. In effect the "interaction energy" discussed by Wyman (40) is given by $RT \ln c$. It should be distinguished from the "energy of quaternary constraint" which would be given by $RT \ln L$. Values of L and c can also be determined by kinetic methods (41, 42), and values of L can be determined from subunit dissociation data (43). The relationship of the Hill plot to other models has also been discussed (44).

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THE MINIMUM UNIT OF COOPERATIVITY

While the equations cited in the preceding section to describe cooperativity were all based on four oxygen binding sites after the work of Adair, models with fewer sites also give cooperative behavior. Indeed, a dominant idea of the 1960s was the action of heme sites in pairs, such that two-chain dimers were the principal functional units, either as free dimers in solution or as pairs of dimers in intact tetrameric hemoglobin (1, 5, 45, 46). This idea, known as the "dimer hypothesis," arose principally from observations with solutions of salts and other agents that cause dissociation of hemoglobin into subunits. For example, Rossi-Fanelli et al (47) reported dissociation of both oxy- and deoxyhemoglobin largely to dimers in 2 M NaCl on the basis of light scattering and sedimentation data. However, 2 M NaCl had only a slight effect on oxygen binding properties (48). Therefore, the hypothesis was advanced that interactions within dimers are highly cooperative, with a Hill constant near 2 and with only slight cooperativity arising from further interactions between dimers. The fact that the oxygen binding maintained a Hill coefficient near 3, although the hemoglobin was believed to be dissociated into dimeric units containing only two oxygen binding sites, created a complication referred to as the "salt paradox," because the Hill constant can never exceed the number of binding sites (49). The paradoxical aspects of the dimer hypothesis were removed, however, with the observation that dissociation of deoxyhemoglobin is less pronounced in 2 M NaCl than oxyhemoglobin (50, 51), and the dimer hypothesis prevailed.

The first major challenge to the dimer hypothesis arose from kinetic studies. The cooperative binding of ligands to hemoglobin revealed by the sigmoidal oxygen binding curve can be expressed in kinetic terms. For example, the ligand

carbon monoxide (CO) binds to deoxyhemoglobin "slowly" ($k \approx 1.5 \times 10^5$ M^{-1} sec^{-1}) in rapid mixing studies; however, if CO-hemoglobin receives a flash of light that photodissociates one CO molecule, it recombines "rapidly" ($k \approx 6 \times 10^6$ M^{-1} sec^{-1}) (4). The flash experiment presumably traps the partially saturated molecule in the *R* state, whereas the mixing experiment reflects the *T* state. Cooperativity is also reflected in an acceleration of the apparent rate of CO binding in the mixing experiment (4). With the isolation of individual α and β chains by the *p*-mercuribenzoate (PMB) method (52), their high affinity and rapid rate of binding of CO, as well as their spectral properties (26, 53), indicated that the isolated chains are in the *R* state.

Rapidly reacting material could also be detected in CO-hemoglobin subjected to a large flash that fully dissociated the CO (54). In this case, slowly reacting hemoglobin was the principal product, but a small fraction of rapidly reacting material persisted which was concentration dependent. At concentrations near μM (heme) the rapid component approached 50% (55). Since dimers were believed to be cooperative units (and presumably slowly reacting) and since early studies on hemoglobin with the ultracentrifuge scanner system gave evidence of dissociation of hemoglobin into monomers in very dilute solutions (56), Antonini and co-workers (45, 57, 58) attributed the rapidly reacting material in dilute hemoglobin solutions to free monomers. However, certain difficulties attended this interpretation. Combined flash and flow experiments on hemoglobin and NO-hybrids (59) were incompatible with interactions of subunits within pairs. Also, ultracentrifuge results indicated that dissociation to monomers occurred at concentrations at least an order of magnitude below the concentrations at which rapidly reacting material appeared (27).

The difficulties were resolved in 1969, when Edelstein & Gibson (60) measured the fraction of rapidly reacting material in parallel with dissociation using the ultracentrifuge scanner. The appearance of rapidly reacting hemoglobin was found to correlate closely with dissociation of tetramers into dimers, not formation of monomers. Thus the rapid component was identified as dimeric hemoglobin, presumably $\alpha\beta$ dimers (28), and serious doubt was cast on the dimer hypothesis. In fact, monomers are not produced to any measurable extent under the experimental conditions used (60–63). Identification of dimers as noncooperative raised additional questions about the salt effect. Evidently the extent of dissociation in 2 M NaCl had been overestimated by failure to take into account preferential interactions between the hemoglobin and the water (60, 61, 64). The results of Kellett indicated that after the proper corrections, no dissociation was detectable for deoxyhemoglobin in 2 M NaCl even at concentrations in the μM (heme) range (64). The possibility of dissociation of deoxyhemoglobin at a different plane than for oxyhemoglobin to produce cooperative dimers was briefly considered (62, 65), but trapping of the dimer formed from deoxyhemoglobin by pH jump experiments revealed that it too was rapidly reacting with CO (66). A similar conclusion was reached in studies on the dithionite reaction at low concentrations (67). Hybridization experiments supported the view that oxy- and deoxyhemoglobin dissociate at the same plane (68). Similar experiments have been used to measure the rate constant for dissociation

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of deoxyhemoglobin tetramers into dimers (69). Finally, it was possible to find conditions where deoxyhemoglobin dissociated into dimers, and noncooperative oxygen binding equilibria were found (70, 71).

The conclusion that tetrameric hemoglobin dissociates to dimers with spectral, kinetic, and equilibrium properties resembling isolated chains or the R state means that as a thermodynamically linked system, hemoglobin and its subunits could be described simply by the scheme

11.

where $K_{4,2}^{T}$ and $K_{4,2}^{R}$ refer to tetramer-dimer dissociation constants for the T and R states, respectively; L describes the $T \rightleftharpoons R$ equilibrium, $L = (T)/(R)$; and r refers to $\alpha\beta$ dimers in the R state. According to this scheme, quaternary constraint arises entirely from interactions between dimeric units. Since $K_{4,2}^{R}$ can be readily measured and has a value near μM for CO-hemoglobin (60–63, 72, 73), a method of determining $K_{4,2}^{T}$ would permit an independent estimate of L, since

$$L = K_{4,2}^{R}/K_{4,2}^{T} \qquad 12.$$

A method for determining $K_{4,2}^{T}$ was developed by Thomas & Edelstein (43, 74) based on the variation of the affinity of hemoglobin for CO with hemoglobin concentration. Plots of log $(CO)_{1/2}$ vs log (heme) are linear with a slope of 0.25 and yield $K_{4,2}^{T}$ from the intercept. A value of $K_{4,2}^{T} = 3 \times 10^{-12}$ M was obtained for 0.1 M phosphate, pH 7. When combined with values for $K_{4,2}^{R}$, a value of $L = 6.7 \times 10^{5}$ was deduced. Addition of 2 M NaCl produced an increase in $K_{4,2}^{T}$ of about twentyfold, roughly the same increase as is produced in CO-hemoglobin by NaCl (51, 62, 64, 75). Thus the reason 2 M NaCl has little effect on oxygenation, in spite of some increase in the dissociation constant of oxyhemoglobin, is that a similar increase in the dissociation constant of deoxyhemoglobin compensates to leave L largely unaltered. The method of measuring $K_{4,2}^{T}$ has also revealed that the Bohr effect coincides with changes in $K_{4,2}^{T}$ (74). Oxygen affinity increases with pH by destabilizing the T state to decrease L, presumably by breaking salt bridges identified by Perutz (76) and his co-workers (see following section).

Since hemoglobin consists of tetramers in equilibrium with noncooperative dimers (in the R state), the complete description of ligand binding in the formulation of the two-state model takes the form (43)

$$Y = \frac{\alpha(1+\alpha)^3 + Lc(1+\alpha)^3 + LK_{4,2}^{T}\alpha(1+\alpha)/2[\mathrm{Hb}_2]}{(1+\alpha)^4 + L(1+c\alpha)^4 + LK_{4,2}^{T}(1+\alpha)^2/2[\mathrm{Hb}_2]} \qquad 13.$$

where $[\mathrm{Hb}_2]$ refers to the concentration of dimers and can be obtained for any total concentration (heme) from the quadratic equation

$$4[\mathrm{Hb}_2]^2\left\{\frac{(1+\alpha)^4}{LK_{4,2}^{T}} + \frac{(1+c\alpha)^4}{K_{4,2}^{T}}\right\} + 2[\mathrm{Hb}_2](1+\alpha)^2 = (\mathrm{heme}) \qquad 14.$$

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Evaluation of this equation reveals that for certain systems (high L) cooperativity should increase with dilution of hemoglobin (43), and results on hemoglobin Kansas support this prediction (J. O. Thomas and S. J. Edelstein, unpublished results). This formulation also demonstrates that L can be altered by changes in either $K^R_{4,2}$ or $K^T_{4,2}$. For example, the Bohr effect involves changes in $K^T_{4,2}$, while several other systems involve changes in $K^R_{4,2}$. Cat hemoglobin has a value of L about tenfold higher than most other mammalian hemoglobins due to a tenfold higher value of $K^R_{4,2}$ (37, 77). Similarly, hemoglobin Kansas has an elevated L value due to a high $K^R_{4,2}$, about 10^{-4} M (33). In contrast, hemoglobin Chesapeake has an unusually low L value due to a low $K^R_{4,2}$ (78). These differences in dissociation can also be related to the binding of haptoglobin (79) since haptoglobin reacts only with $\alpha\beta$ dimers (80).

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DEDUCTIONS FROM STRUCTURAL STUDIES

The work of Perutz and his associates has now revealed a great deal of information on the precise arrangement of atoms in horse and human deoxyhemoglobin and oxyhemoglobin (actually methemoglobin, since the crystals are oxidized during the measurements) as well as many mutant forms and chemically altered variants (see 76 and 81–83, which review many aspects of this large body of work). Since these papers are synoptic and interpretive, only the main features of the deductions will be reviewed here. However, it should be noted that since the conclusions are based on comparisons of the structures of deoxyhemoglobin and methemoglobin, whose interconversion is only weakly cooperative, some important modifications of the conclusions may be required when the comparison of deoxyhemoglobin to an Fe^{2+} liganded form, such as CO-hemoglobin, is available. The basic difference between the T (deoxy) and R (oxy-met) structures deduced from low resolution data (84) has been supported by data at higher resolution (85, 86). The $T \leftrightarrow R$ transition involves a rotation of $\alpha\beta$ units with respect to one another that maintains the α_1–β_1 interface but alters the α_1–β_2 interface, with the most marked rearrangement involving an increase in distance between the β chains upon deoxygenation (see also 87). It is the separation of β chains that accommodates organic phosphates (88, 89) to depress oxygen affinity by stabilizing the T state (8, 90). Interaction of 2,3-diphosphoglycerate (DPG) with β chains had been proposed from studies on chemically modified hemoglobins (91, 92). Some additional Bohr effect is also caused by DPG binding (93–96).

The conformation of the T state permits formation of several salt bridges (76) with the C-terminal residues, Arg 141α and His 146β, participating. These salt bridges provide the proton binding sites in the T state that produce the Bohr effect. The involvement of the residues has been demonstrated by the measurements of Kilmartin and his co-workers on the oxygen binding properties and Bohr effect of carboxypeptidase-digested hemoglobins (97–99) and carbamylated hemoglobin (100). The later derivatives have also permitted a description of the mechanisms of CO_2 binding (101) that has greatly clarified the physiological processes involved (9). Since the salt bridges in the T state stabilize the protonated

forms, transition to the R state breaks the salt bridges, the relevant pK values are lowered, and protons are released. Similarly at high pH the salt bridges are broken by deprotonation even in the T state, accounting for the increased affinity for oxygen with increasing pH (alkaline Bohr effect).

Among other important structural features of hemoglobin is the two-state arrangement of the α_1–β_2 interface. The contact is "dovetailed" (82) so that a hydrogen bond linking Tyr 42α and Asp 99β in the T state is replaced by a bond between Asp 94α and Asn 102β in the R state. The position of the iron with respect to the heme plane has also come under close scrutiny. Work of Hoard and his associates on model compounds (102, 103) led them to propose that high spin iron, as occurs in deoxyhemoglobin and aquo- and fluoro-methemoglobin, would be too large in radius to be accommodated within the heme plane; low spin iron, as occurs in oxyhemoglobin and cyanide- and azide-methemoglobin would be reduced in radius and could be accommodated within the heme plane. This situation has been partially verified for the iron atoms in aquo-met hemoglobin, which are 0.3 Å from the heme plane (76), and measurements on deoxyhemoglobin indicate a displacement of the iron from the heme plane of 0.75 Å (76).

Apart from the structural data, Perutz (76, 81) has made a number of interpretive proposals on the detailed mechanisms of oxygen binding. While they are of heuristic value, not all are in agreement with experimental evidence. The proposals can be divided into two areas: (*a*) the sequence of events in the binding of ligands to the α and β chains and their relation to the breaking of the salt bridges and the $T \rightarrow R$ transition, and (*b*) the role of out-of-the-plane to planar transition of the iron upon oxygenation as a trigger in the $T \rightarrow R$ transition.

Concerning the first area, Perutz (76) argues that the sequential events in oxygenation are connected by a structural interaction involving the four iron atoms and the four penultimate tyrosine residues. The binding of oxygen is postulated to occur first to the hemes of the α chains and coincides with expulsion of the penultimate α chains' tyrosines from pockets between the F and H helices with the attendant breaking of the salt bridges of α chains and release of Bohr protons. According to Perutz, binding occurs first to the hemes of the α chains because access to the hemes of the β chains in deoxyhemoglobin is restricted by the γ methyl group of Val β67. As each subunit binds ligand, a tertiary conformational change (designated $t \rightarrow r$) is postulated to occur to give an R-like subunit in the T quaternary structure. When several subunits have bound ligand, a transition to the R quaternary structure occurs with a release of organic phosphates and additional Bohr protons. The binding of ligands to the β chain occurs with breakage of the internal salt bridges of the β chain. It now appears highly unlikely that all ligand binding occurs with precisely this sequence of events, although some elements of the proposal may be applicable, as will be apparent from the physical-chemical studies discussed in the remainder of this review. In addition Szabo & Karplus (38) start with the same assumptions as Perutz but arrive at different conclusions concerning the sequence of binding events.

Concerning the role of the iron, Perutz (81) argues that the diminished affinity of the T state for ligands is related to a reciprocal effect between spin state

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of the iron and interactions with the globin mediated by the imidazole ring of His F8. The basic argument has been related to experiments on methemoglobin (104–107) in which an $R \rightarrow T$ transition may occur upon addition of inositol hexaphosphate (IHP), the organic phosphate most effective in stabilizing the T state (8). Spectral changes accompanying addition of IHP to methemoglobin are interpreted as reflecting an increase in the high spin character of the iron that coincides with the $R \rightarrow T$ transition. Thus the conformation of the T state is visualized as applying a tension at the heme irons which heightens their high spin character and contributes to the diminished affinity of the T state for ligands. When a ligand is bound, the iron is transformed to a low spin planar form which, Perutz argues, triggers a $t \rightarrow r$ conformational change in that subunit. This hypothesis is also supported by the studies of Banerjee and his co-workers on ligand binding to certain valence hybrids (108) and methemoglobin (109); a quantitative analysis of these studies has been presented by Szabo & Karplus (110). However, recent results by Hensley et al (111) are at variance with these conclusions. Many indices of conformation such as tetramer-dimer dissociation and SH reactivity are found to be perturbed by IHP, but in a manner largely independent of spin state. Moreover studies by Edelstein & Gibson (112) on the effect of IHP on the redox reaction of hemoglobin indicate that a major consequence of binding IHP is the enhancement of α–β chain nonequivalence in redox potential. In addition, a quantitative analysis of the effects of the redox reaction which incorporates chain nonequivalence indicates that only a fraction of the molecules of methemoglobin in the presence of IHP are in the T state (112). On the basis of these results, the movement of the iron is not considered to be a major factor in the $T \rightarrow R$ transition, so there is at least some degree of uncertainty concerning the validity of the Perutz hypothesis on the role of spin state changes in the iron as a trigger of the conformational transition. Because work on this subject is not yet sufficiently definitive to permit a succinct summary and space limitations preclude a discussion of all the experimental evidence and interpretations, a full exposition of this topic must await a future review. The papers already cited (104–112) discuss the issues in considerable detail and little further clarification can be added at this time.

DETECTION OF THE $T \leftrightarrow R$ TRANSITION

Evidence for a $T \leftrightarrow R$ equilibrium in hemoglobin began accumulating with Gibson's observation (54) of the fast reacting form, referred to as Hb*, in partial flash experiments on carboxyhemoglobin. The rate of CO binding to Hb* is 30–40 times higher than the rate of CO combination with deoxyhemoglobin in rapid mixing experiments, and the spectrum of Hb* is depressed and broadened in the Soret region. Spectral and kinetic properties similar to Hb* were obtained for isolated α and β chains of hemoglobin (26), suggesting that the Hb*-type properties represented an unliganded or deoxy R state. This line of reasoning was supported by studies on carboxypeptidase-treated hemoglobins which also share the spectral and functional properties of the R state in the deoxy form (in the absence of

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organic phosphates), but which can be switched towards a normal T state deoxy form (typical Soret spectrum, reduced combination velocity with CO, cooperativity in oxygen binding) by addition of IHP (104, 113–117). While a deoxy T state can be produced in these variants in the presence of organic phosphates, the loss of a salt bridge lowers the L value, as seen by higher than normal oxygen affinity. A higher than normal rate of CO combination is also observed and cooperativity is pH dependent, decreasing with increasing pH (96, 98, 113), corresponding to the properties associated with the left side of the bell curve of n vs log L (Figure 1). Broadly similar findings are obtained with mutant forms of hemoglobin such as hemoglobin Hiroshima (118), hemoglobin Bethesda (119), hemoglobin Kempsey (120), and hemoglobin Chesapeake (121).

In the case of hemoglobin Kansas, a low affinity variant, addition of IHP appears to maintain the molecule in the T state, as revealed by NMR studies of Ogawa et al (122). Hopfield et al (123) attempted to explain its ligand binding kinetics on the basis of this observation, although the situation appears to be complicated by specific effects of the mutation on chain heterogeneity reported by Gibson et al (124). In addition, organic phosphates would be expected to perturb the tetramer-dimer equilibrium of hemoglobin according to the hypothesis of Ogawa and Hopfield et al (122, 123), although Gibson et al (124) reported that no such effect was observed. A thirtyfold reduction in $K_{4,2}$ was observed upon addition of IHP ($K_{4,2}$ changes from 4×10^{-4} M to 1.5×10^{-5} M) by Hensley and Edelstein (unpublished results) in sedimentation equilibrium experiments with an online computer system (73, 125), but only in 0.3 M bis-tris buffer, the conditions of the NMR experiments. In buffers used by Gibson et al (124) the effect of IHP is negligible. Thus the various results may simply reflect different conditions and not a fundamental discrepancy.

Work with valence hybrids in which either the α or β chains are prepared in the ferric form (126, 127) has also supported the view of an organic phosphate-dependent T-R equilibrium. Ogawa & Shulman (128) identified NMR peaks characteristic of either oxy- or deoxyhemoglobin that predominated in the valence hybrids in the absence and presence of phosphates, respectively (see also 129–131). Moreover, the addition of organic phosphates produced a slow change from a mixture of slowly and rapidly reacting components in CO combination experiments to slow material only, indicating a $R \rightarrow T$-type transition (132, 133). Similar conclusions have been reached by Lindstrom et al (134) with M-type hemoglobin variants in which one chain is naturally in the ferric form [see review by Ranney et al (135)]. Evidence for a T-R equilibrium permits an explanation of the effect of organic phosphates on valence hybrids without recourse to sequential models as had been proposed (136).

Thus the presence of an $R \leftrightarrow T$ transition has been demonstrated in many hemoglobin variants, and evidence has also been obtained in normal hemoglobin. Cassoly & Gibson (137) have recently demonstrated the production of a form that is fast reacting with CO in experiments on binding of NO and CO mixtures to hemoglobin. Earlier, combined flash photolysis and stopped flow mixing experiments of Gibson & Parkhurst (59) revealed that the fast reacting form produced with a

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partial flash appears after about three molecules of CO are bound. Development of a kinetic formulation by Hopfield et al (41) of the two-state model indicated that this behavior is in good agreement with the predictions of the model. [Other deductions concerning oxygen binding are complicated by chain heterogeneity (138) discussed below.] In addition, the release of organic phosphate (which binds preferentially to the T state) upon ligand binding occurs with a lag with respect to CO binding, as determined in studies with fluorescent analogs (139, 140). Release is earlier in variants with higher affinity, where lower L and earlier $T \rightarrow R$ transitions are expected (140). The binding of p-mercuribenzoate to the reactive SH group of hemoglobin, $\beta 93$, also appears to coincide with the $T \rightarrow R$ transition (141). Earlier workers who concluded a coincidence of p-mercuribenzoate binding and ligand binding (142) were misled by the narrow range of conditions examined.

Since salt bridges are present in the T state and absent in the R state, a release of protons associated with the salt bridges (the origin of the alkaline Bohr effect) could be expected to accompany the $T \rightarrow R$ transition. However, pivotal kinetic studies of Antonini et al (143) and Gray (144) indicate a linear release of protons with CO binding, not a lag as would be expected for a parameter that reflects the $T \rightarrow R$ transition. These findings have had a major impact on formulations of cooperativity and are at least partially responsible for Perutz's (76) proposition of a $t \rightarrow r$ transition for each subunit as ligand is bound or a salt bridge is broken. Recently a new light has been cast on this subject by Olson & Gibson (145), who find that proton release does lag behind CO binding when IHP is present.

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EVIDENCE FOR NONEQUIVALENCE OF THE α AND β CHAINS

Basic Observations

An important recent development in hemoglobin research is the discovery of significant differences in the ligand binding properties of the α and β chains, principally by Gibson and his co-workers. The first evidence for chain nonequivalence came in studies on methemoglobin (146, 147). For example, the reaction of methemoglobin with azide is about sixfold more rapid with β chains than α chains, with the faster reacting chain identified by spectral studies on isolated chains and intact hemoglobin. Similar results are obtained for the dithionite reaction. Nitrite and thiocyanate also react more rapidly with β chains, although α and β rates are about the same for cyanide and fluoride (146). Chain nonequivalence for imidazole has also been reported (148, 149). The first major α–β difference for deoxyhemoglobin was found for n-butylisocyanide (BIC) by Olson & Gibson (150). This ligand binds to and dissociates from β chains more rapidly than α chains (151, 152). Identification of the chains was made principally by (a) a comparison of properties with isolated chains and p-mercuribenzoate-reacted β chains (151), (b) NMR studies on partially saturated mixtures (153) which indicated preferential disappearance of a resonance identified with β chains in deoxyhemoglobin (154), and (c) binding to valence hybrids (133, 152). While α and β properties are almost equal at pH 9 or at pH 7 in low ionic strength, addition of salt or organic phosphates leads to the preferential binding of BIC to β chains,

principally through a reduction in the rate of binding to α chains (155). In the presence of IHP, chain differences are so great that cooperativity is abolished. Excellent agreement with these kinetic studies was obtained by temperature jump relaxation methods (156). The conclusion of preferential binding of BIC to β chains has been challenged by Huestis & Raftery (157) on the basis of NMR measurements with a ^{19}F-trifluoroacetone derivative at the $\beta 93$ position, but it may be possible to reconcile the disparate observations (see below).

Concerning more traditional ligands, some chain differences in CO binding in the presence of organophosphates were detected by Gray & Gibson (158). Studies on the valence hybrids suggest that in this case α chains represent the rapid component (133). Recently Gibson (138) has demonstrated that oxygen binding kinetics closely parallel those of BIC. For example, in oxygen pulse experiments in which a solution of oxygen is mixed with a solution containing hemoglobin and dithionite, the oxygen binds to hemoglobin but dissociates from partially saturated intermediates due to combination with dithionite. Under these conditions oxygen binds to and dissociates from β chains rapidly. The rate constants for the α chains are so low as to suggest that oxygen binds almost exclusively to β chains in the T state, although the affinity of α chains for oxygen may actually be higher than for β chains due to a compensating dissociation rate (138). This scheme should also bear an important relationship to temperature jump relaxation studies on the kinetics of oxygen binding to hemoglobin (159). Chain differences in oxygen binding affinity were also suggested by Ogata & McConnell (160–163) on the basis of spin-label studies. NMR studies of Lindstrom & Ho (164) indicate a preferential binding of oxygen to α chains. Studies by Henry & Cassoly (165) on NO binding indicate a chain heterogeneity in which NO binds preferentially to α chains (see also 166).

The discovery of conditions under which chain differences are readily apparent has also permitted a more detailed examination of the release of Bohr protons (145). When BIC binding and proton release are compared under conditions where binding to β chains is much more rapid than binding to α chains, about 20% of the proton release is associated with rapid binding to β chains, whereas 80% of the proton release is associated with the slower binding to α chains. This observation appears to be in conflict with the finding of Kilmartin et al (97, 100) that 50% of the Bohr effect is associated with the imidazole groups of His 146β. However, greater association of protons with the α chains is consistent with a larger Bohr effect in $\alpha_2^{II}\beta_2^{III}$ valence hybrids (126), although Brunori et al (127) did not observe this difference. In addition, a larger Bohr effect for M-type variants with a ferrous α chain is observed than for the variants with a ferrous β chain (135). However, since experiments with solutions containing IHP indicate that proton release is coupled to the $T \rightarrow R$ transition (145), the 20% proton release associated with rapid binding to the β chains may simply reflect the extent to which a $T \rightarrow R$ *transition* occurs with the binding of two molecules of BIC (167). Although the proton release appears to be accommodated by this explanation, there is still some discrepancy concerning release of a fluorescent 2,3-diphosphoglycerate analog, which lags behind ligand binding to a greater extent than proton release. Thus a completely self-consistent explanation of the time course of proton release and organic phosphate release is not yet apparent.

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Consequences of Chain Nonequivalence

With the recognition that distinct properties of the α and β chains must be taken into account for a complete description of ligand binding, the binding expression of the two-state model, equation 7, must be expanded to include contributions of each chain. If a, a normalized binding parameter for the R state, is reserved for α chains $[a = (X)/K_R^\alpha]$, a companion term, b, can be defined for the β chains in the R state $[b = (X)/K_R^\beta]$. The properties of the α and β chains in the T state can then be expressed in terms of c_α and c_β, where $c_\alpha = K_R^\alpha/K_T^\alpha$ and $c_\beta = K_R^\beta/K_T^\beta$. With these terms it is possible to derive distinct binding expressions for the α and β chains, Y_α and Y_β, respectively (112, 167)

$$Y_\alpha = \frac{a(1+a)(1+b)^2 + Lc_\alpha a(1+c_\alpha a)(1+c_\beta b)^2}{(1+a)^2(1+b)^2 + L(1+c_\alpha a)^2(1+c_\beta b)^2} \qquad 15.$$

$$Y_\beta = \frac{b(1+b)(1+a)^2 + Lc_\beta b(1+c_\beta b)(1+c_\alpha a)^2}{(1+a)^2(1+b)^2 + L(1+c_\alpha a)^2(1+c_\beta b)^2} \qquad 16.$$

The complete binding properties are then described by

$$Y_{\text{total}} = (Y_\alpha + Y_\beta)/2 \qquad 17.$$

Equation 17 is formally equivalent to an equation derived by Ogata & McConnell (160) and a generating function of Szabo & Karplus (38) but has the advantage that α and β saturation are separated so that each can be evaluated independently, as is required in certain types of analysis (112, 147, 167). Introduction of α–β nonequivalence also alters the interpretation of the asymptotes of the Hill plot (Figure 2) and the equation for L (equation 10).

Since α–β differences in affinity for ligand can occur in either the T or the R state, it is important to determine which state is responsible for any given observation of chain differences. For conditions where a high value of L applies (such as in the presence of organic phosphates for hemoglobin), only chain differences in the T state can give rise to preferential binding to one of the chains in partially saturated solutions (167). This situation arises from the fact that at high L values the T state exists in various degrees of saturation, and chain differences can be revealed. Since the R state only predominates after about three of the four ligand molecules are bound, the possibility of a range of degrees of saturation for the R state in which α–β differences could be revealed does not exist. (At the other extreme, low values of L, only chain differences in the R state can be revealed; depending on the nature of the experiment, kinetic measurements may reveal chain differences in either state.) Thus, the preferential binding of oxygen to α chains in the presence of IHP as determined by NMR measurements (164) must reflect a higher affinity for oxygen of α chains compared to β chains in the T state.

In the case of BIC, although the combination velocities are similar to oxygen, the NMR data indicating preferential binding to β chains (153) must be interpreted as preferential binding to the β chains in the T state. This view is supported by studies of McDonald, Hoffman & Gibson (168) on manganese-iron hybrids in which

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the manganese chain is effectively locked in the T state (169). With $\alpha^{Fe^{2+}}\beta^{Mn^{2+}}$ only extremely slow binding occurs with BIC. Thus, hemes of the α chains in the T state appear to be relatively inaccessible to BIC. Even the behavior of stripped hemoglobin in BIC binding can be explained without recourse to binding of α chains in the T state. In this case the binding data are highly cooperative and the kinetics relatively homogeneous (155) due to the fact that the transition to the R state occurs at low levels of saturation and the properties of the R state (in which chain differences are relatively minor) dominate the behavior (167). These explanations of chain differences can also be extended to the observations of Huestis & Raftery (157) which were interpreted in terms of preferential binding of BIC to α chains. Examination of the parameters of the two-state model corresponding to the conditions of the measurements (167) indicates that what was interpreted as an indicator of binding to β chains (changes in the NMR signal of ^{19}F-trifluoroacetone attached to the $\beta93$ position) is more likely a monitor of the $T \rightarrow R$ transition, i.e. an indicator of the state function, $\bar{R}$, (167) where

$$\bar{R} = \frac{(1+a)^2(1+b)^2}{(1+a)^2(1+b)^2 + L(1+c_\alpha a)^2(1+c_\beta b)^2} \qquad 18.$$

Therefore, the fact that changes in the NMR signal are not linear with saturation is simply an indication that the L value is high and the transition to the R state occurs when about three molecules of ligand are bound.

GENERAL CONCLUSIONS AND CURRENT ISSUES

The availability of structural models for both deoxyhemoglobin and liganded hemoglobin, together with evidence that indicates a two-state model with α–β differences as the basic mechanism for cooperative oxygen binding, have brought hemoglobin research to the point where questions are now phrased in precise physical-chemical terms. One current concern is the extent to which energies reflected in the values of L and c can be identified with particular structural elements in the hemoglobin molecule. Hopfield (170) has suggested three ways in which the affinity of the T state for ligand could be reduced compared to the R state: (*a*) a "direct bond" model in which a chemical interaction of the iron in the T state opposes ligand binding; (*b*) an "indirect bond" model in which energy at a bond at some distance from the iron is set in opposition to ligand binding; and (*c*) a "distributed model" in which many low energy contacts influence binding. Hopfield favors the third alternative and extends it to include a formulation in which interaction energy may vary linearly with the displacement of the iron from the heme. In the mechanism detailed by Perutz (76, 81) involving the breaking of salt bridges accompanying ligand binding, the salt bridges would represent an indirect bond, the second alternative of Hopfield (170). Perutz proposed this mechanism partly to account for the linear release of Bohr protons with ligand binding (143, 144), but the studies already discussed on solutions containing IHP (145) in which a lag is observed cast doubt on the general validity of the coupling of ligand binding and proton release.

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Szabo & Karplus (38) have extended the Perutz point of view to a quantitative analysis of ligand binding. The model is based on a set of intrinsic ligand binding constants for α and β chains which are potentiated by coupling to salt bridges to give rise to the low affinity of deoxyhemoglobin. The model assumes a tertiary transition that includes release of Bohr protons with ligand binding and thus may be difficult to reconcile with data indicating a lag in proton release in the presence of IHP (145). These kinetic experiments thus take on major significance. Several other interpretive papers and new formulations of cooperativity have also appeared recently (171–175).

Although the kinetics of CO binding can be accommodated by a kinetic version of the two-state model to a first approximation (41), small deviations in the constants for the first to third binding events have consistently appeared (59, 139, 140) and warrant further investigation. The two-state model therefore provides a more nearly perfect representation for mutant forms with a low L value where high binding rates of the R state apply after the slow CO binding rate for the first site of the T state (118–121). Kinetic formulations of the two-state model for oxygen binding where α–β differences must be included have not yet been reported. In the treatment by Hopfield et al (41), the $T \leftrightarrow R$ transition was assumed to be fast compared to ligand binding. However, studies on variants with a low value indicate that the $T \leftrightarrow R$ transition is relatively slow (117, 133). Since a value of L near unity applies to the $T_3 \leftrightarrow R_3$ transition, a rate-limiting conformational transition might be involved for binding of certain ligands to normal hemoglobin.

The structural reason for the disparate behavior of different ligands remains obscure and deductions from structural models do not appear adequate to explain the phenomena. One intriguing pattern that has emerged is that for the ligands NO, CO, O_2, and BIC, the higher the affinity of the ligand for hemoglobin, the greater the discrimination in favor of α chains over β chains in the binding rates (133, 138, 145, 150, 158, 165).

Ligand Affinity (highest to lowest)	Relative Binding Rates (for deoxyhemoglobin)
NO	$\alpha \gg \beta$
CO	$\alpha > \beta$
O_2	$\alpha < \beta$
BIC	$\alpha \ll \beta$

Some understanding of the details of ligand binding may be realized in the mechanistic studies of Austin et al (176). Interest in the mechanism of ligand binding has also been stimulated by recent studies of photosensitivity of CO binding (177–180). The work of Alpert et al (181) with a laser flash system has revealed a very rapid conformational change in the nanosecond range that may require incorporation in any complete kinetic scheme.

Hemoglobin also provides an ideal system for the development and testing of structural principles with broad implications for protein chemistry. A precise description of the energetics of the interfaces should be an ideal testing ground

for potential functions related to amino acid side chain interactions. At present, there is not even a satisfactory description of why isolated β chains associate to the tetramer level while isolated α chains remain unassociated. However, Tainsky & Edelstein (182) have provided a tentative explanation for the unusual stability of β_4 tetramers in strong salt solutions (183) based on particular interactions at the subunit interfaces. Perutz (184) has recently offered an attractive explanation for species differences in alkaline denaturation rates based on differences at the interfaces. Marked differences in the aggregation properties of individual α and β globins exist (185) which have not yet been explained in structural terms. Molecules with heme on only one type of chain, semihemoglobins, provide a rich repertoire of properties, depending on which chain contains the heme and the method of preparation [see review by Cassoly & Banerjee (186)]. The structural principles responsible for these varieties have not yet been revealed. Studies on porphyrin-globin preparations (187–190) and modified porphyrins (6) also provide interesting structural parameters that must be incorporated in any comprehensive description of hemoglobin. The spin-labeled heme of Asakura (191) may be particularly interesting in this regard. Recent studies on porphyrins in which the iron is replaced by other metals, including manganese (192–194), cobalt (195–200), and zinc (201), exhibit distinctive features that are likely to contribute important information for a full description of the properties of hemoglobin. Improved physical-chemical approaches also promise to open new avenues of investigation for hemoglobin. Tritium exchange studies by Englander and his colleagues (202, 203) are providing a unique perspective on the conformational dynamics of hemoglobin (see also 204). Improvements are occurring in calorimetric measurements during ligand binding (205, 206). Resonance Raman spectroscopy is emerging as a very powerful method for heme proteins (207–209). Yamamato et al (209) have interpreted their Raman spectra of oxyhemoglobin in terms of iron that is formally in a low spin ferric state. Important variations and new principles may also emerge from studies on nonmammalian hemoglobins. What is certain about the trends in improved methodology is that in spite of the large body of information already accumulated, the ramifications of hemoglobin research are likely to continue to actively engage investigators for some time.

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