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Extensions of the Allosteric Model for Haemoglobin

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So far it has not been possible to distinguish experimentally between two possible models for cooperative ligand binding by haemoglobin. Numerical analysis, however, yields a unique value for the allosteric constant showing that only the two state model can account for the behaviour of the protein.

A GREAT deal of information is now available about the structure of haemoglobin, largely from the X-ray studies of Perutz[1]. The differences in quaternary conformation of the liganded and unliganded forms of haemoglobin have been specified and many of the residues which are involved in the conformational change have been identified. These structural studies provide a clear picture of many of the atomic movements accompanying ligand binding. Certain dynamic aspects of the cooperative ligand binding by haemoglobin, however, remain uncertain. According to the allosteric model by Monod *et al.*[2] cooperativity would arise simply from the adjustment of an equilibrium between the two conformational states observed by Perutz. But alternative models have been described, notably the sequential model proposed by Pauling in 1935[3] and favoured by Koshland *et al.*[4] in which conformational changes occur in steps during ligand binding. The two state and sequential models differ in several details, but from the point of view of fundamental processes, the chief distinction concerns the existence of conformational equilibria. The treatment by Monod *et al.* requires a pre-existing conformational isomerization equilibrium which is perturbed by ligand. The Koshland approach is based on induced fit whereby changes in conformation only occur after ligand binding.

Several experimental approaches have been reported and interpreted to exclude one model and indirectly imply the other; the most significant fit into two categories: (1) tests for the deviations from linearity in the response of some structural parameter to the addition of ligand as predicted by the two state model in certain conditions; (2) experiments which seek to detect the conformational equilibrium by attempting to measure the small amounts of one conformation postulated to exist by the two state model, in conditions which greatly favour the other. For haemoglobin, a typical experiment of the first type was the spin-label study of Ogawa and McConnell[5]. Linearity of the spin-label change to oxygenation was reported, whereas the behaviour predicted from calculations with the two state model was distinctly non-linear. The results were therefore interpreted as implying a sequential model. The second type of experiment is illustrated by work with the haptoglobin–haemoglobin system. Haptoglobin binds very tightly to oxyhaemoglobin but generally not at all to deoxyhaemoglobin[6,7]. Because the conformational isomerization of the two state model requires some small amount of the oxy-like material in the deoxyhaemoglobin solutions (which should bind haptoglobin) the failure to observe binding has been cited as evidence against the two state model[8]. Conclusions of both types of experiment, however, rely heavily on the value

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of the conformational isomerization equilibrium constant used. This constant, L in the nomenclature of Monod *et al.*[2], has been estimated in all cases only by casual fitting. We wish to describe a more rigorous method for evaluating L based on a consideration of the properties of both haemoglobin and its isolated chains. Values of L much larger than earlier estimates are obtained by this procedure, and the revised values bear critically on the several experiments designed to distinguish models.

The second part of this article concerns the incorporation of the new values of L in an analysis of the cooperativity of haemoglobin in various conditions for normal and mutant haemoglobins. Efforts at accommodating this set of ligand binding curves with both the two state and sequential models are described.

Size of Allosteric Constant for Haemoglobin

The basis of the two state model is an equilibrium between the oligomeric states called R and T. The relaxed state, R, displays high affinity for ligand (given by the dissociation constant K_R) and the constrained state exhibits low affinity for ligand (given by K_T where $K_R < K_T$ and $c = K_R/K_T$). The isomerization equilibrium is described by the equilibrium constant L, where $L = [T]/[R]$ in the absence of ligand. The term L is also referred to as the allosteric constant. Monod *et al.*[2] originally reported a value for haemoglobin under standard conditions of $L = 9 \times 10^3$ and $c = 0.014$. The values are obtained[2] by fitting oxygen saturation data to the equation

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

where Y is the fractional saturation and α is a normalized ligand concentration ($\alpha = pO_2/K_R$). A detailed analysis of the fitting of this equation to the oxygen binding properties of haemoglobin yields two important points. First, the values of L and c reported by Monod *et al.*[2] are not unique and widely differing combinations can also represent the data, particularly since K_R is not specified (Fig. 1). Two theoretical curves with differences that fall within usual experimental errors are shown to represent the ligand binding by haemoglobin. The values of L for the two curves differ greatly: 2×10^3 compared with 3×10^5. The second point, however, is that a unique solution to the two state equation for haemoglobin is possible by considering the properties of the isolated chains.

By virtually all criteria, the isolated α and β chains of haemoglobin possess the same, or very nearly the same, conformation as the individual chains in liganded $\alpha_2\beta_2$ haemoglobin. The criteria include spectral similarities[9] and agreement in kinetic properties between chains[10] and the short lived "rapid" haemoglobin observed in the Gibson effect[11]. In the one exception[12], the differences are only minor. Therefore, if chains and the R state are effectively identical, the ligand affinity of the chains is an indicator of the affinity of the R state and defines K_R. Since chains are apparently locked in the high affinity state, equation (1) reduces to equation (2)

$$Y = \frac{\alpha}{1+\alpha} \qquad (2)$$

and at half saturation $\alpha_i = 1.0$. Thus as a consequence of the definition of α a scale for haemoglobin is provided with α_i for chains set at unity and the relative affinity of haemoglobin set accordingly. For example, the affinity of normal human haemoglobin at *p*H 7 is about twenty-four to thirty times less than isolated chains[13]. The α_i must therefore fall in the range of twenty-four to thirty.

With α_i fixed, the values of L and c may be considered. First, the constant L is directly related to α_i. Chains are characterized by $\alpha_i = 1$, so the higher values of α_i observed for native haemoglobin are linked to the relative stability of the T state: the more stable the T state (that is, the larger the value of L), the higher the value of α_i. Because four molecules of ligand are bound by each molecule of haemoglobin, the exact linkage relationship takes the form $L = (\alpha_i)^4$. A more complete development of these relationships will be presented later, when the linkage to subunit interactions will also be considered. With a minimum value of α set at 24, a minimum value of $L = 3 \times 10^5$ is obtained. Any lower value of L will yield a predicted ligand binding curve with an affinity too close to that of chains. With $L = (\alpha_i)^4$, the cooperativity as reflected by the Hill constant, n, is set by c. For $L = 3 \times 10^5$, $n = 2.8$, the usual experimental value for haemoglobin, when $c = 0.01$. Higher values of c lower n and diminish cooperativity.

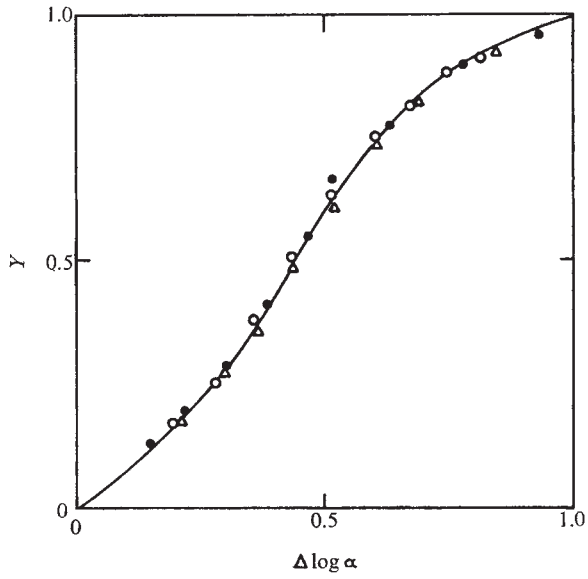

Fig. 1 Representative oxygen binding for haemoglobin. Fractional ligand binding (Y) versus the logarithm of the relative ligand affinity. The points (●) represent a ligand binding curve generated from the empirical Adair equation using values for the four phenomenological equilibrium constants described by Gibson[15]. These values are in good agreement with early gasometric determinations. For these data, zero on the abscissa corresponds to log $pO_2 = 0.6$. Two additional sets of points are provided, calculated with the two state model (equation 1). For the points represented by (△) a value of $L = 3 \times 10^5$ was used. The points represented by (○) are obtained with $L = 2 \times 10^3$. The two theoretical curves are on shifted scales, corresponding to different values for the unspecified parameter K_R. For the curve with $L = 3 \times 10^5$, zero on the abscissa is equal to a value of log $\alpha = 0.9$, and for $L = 2 \times 10^3$, log $\alpha = 0.4$.

In the simple formulation of the two state model, no intermediate states are considered. If intermediate states occur, our analysis still applies in terms of L. But intermediates destabilize cooperative effects and as a consequence the value of c must be diminished to maintain the Hill constant at $n = 2.8$. For example, if one intermediate state, I (half R, half T), is added to the two state formulation, the proper equations are easily derived[14] and a new parameter L_1 appears ($L_1 = [I]/[R]$ in the absence of ligand). In this case, a good representation of the haemoglobin saturation curve is obtained with $L_1 = 750$ and $c = 0.001$. The value of L remains fixed at 3×10^5. This particular set of parameters was selected, since the kinetic data of Gibson[15] suggest a value of c close to 0.001. The presence of an intermediate state would also account for the two stage process implied by Gibson[15]. Thus, the magnitude of an intermediate term, L_1, may prove some measure of the extent to which the two state model is an approximation and inter-

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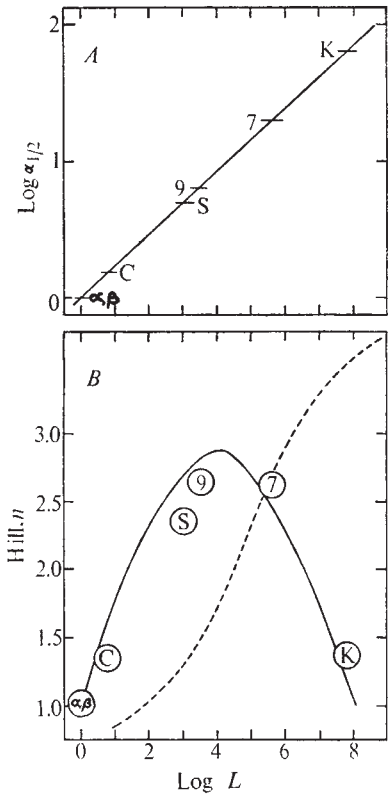

Fig. 2 Composite ligand binding properties for haemoglobin. *A*, Dependence of the logarithm of ligand binding affinity on the logarithm of the allosteric constant. The line was obtained by generating a series of theoretical ligand binding curves with equation (1) with $c=0.01$ and plotting log $\alpha_{\frac{1}{2}}$ versus log L. The straight line resulted. The various examples of haemoglobin: isolated chains ($\alpha+\beta$), Chesapeake (C), stripped (S), normal at *p*H 7 (7), normal at *p*H 9 (9) and Kansas (K), were then located on the affinity curve in terms of their measured properties relative to the isolated chains. *B*, Dependence of cooperativity (the Hill constant, n) on log L. For the series of theoretical curves used to generate log $\alpha_{\frac{1}{2}}$ versus log L in *A*, the overall cooperativity was also estimated. The average value of n, where $n=d \log (Y/1-Y)/d \log \alpha$, between $Y=0.25$ and $Y=0.75$ was calculated. When these values of n are plotted against log L, the bell shaped curve is obtained. The six points circled, representing the examples of haemoglobin, were located horizontally on the curve according to the value of log L indicated in *A*. The vertical position corresponding to cooperativity was determined by the published values from equilibrium measurements (see text). The dashed line represents values of n calculated for the sequential model, using equation (13) of Koshland *et al.*[4]. Values of K_{BB} and K_{AB} were selected to represent the observed affinity and cooperativity of haemoglobin at *p*H 7.0. The general dependence of cooperativity on log L (equivalent to log $K_{BB}{}^6$) was then determined by generating binding curves with different values of K_{BB}.

mediate states may have to be included. However, stripped haemoglobin, which is perhaps more easily interpreted kinetically because factors related to phosphate binding can be ignored, yields values closer to $c=0.01$ (ref. 15) and is therefore less suggestive of intermediates.

Conformational Equilibria and Cooperativity

With the quantitative procedure for obtaining the value of the allosteric constant, L, described earlier in this article, it is now possible to compare the behaviour of haemoglobin in various conditions, as well as mutant haemoglobins, in terms of mechanistic models. One of the most salient features of the two state model is the "buffering" of cooperativity caused by the conformational equilibrium. As first pointed out by Rubin and Changeux[16] cooperativity is relatively independent of L for values of L near $c^{-i/2}$ (where i is the number of binding sites). The overall dependence of cooperativity on L is bell shaped with the Hill constant, n, diminishing at both high and low values of L. This property of the two state model was emphasized by Rubin and Changeux[16] to accommodate the Bohr effect in haemoglobin—the change in affinity with little or no change in cooperativity observed with changing *p*H. The dependence of cooperativity and affinity on L is further explored in this paper encompassing a wider range of haemoglobin ligand binding data.

The general dependence of $\alpha_{\frac{1}{2}}$ and L is illustrated in Fig. 2*A*. The values of log $\alpha_{\frac{1}{2}}$ vary linearly with log L, with $\alpha_{\frac{1}{2}}=1$, corresponding to haemoglobin chains. Haemoglobin at *p*H 7 and *p*H 9 (ref. 17), stripped haemoglobin[19] and the mutant haemoglobins Kansas[19] and Chesapeake[20] can therefore be placed on the line of log $\alpha_{\frac{1}{2}}$ against log L according to their measured affinities. A unique value of L is thereby specified for each haemoglobin. With L fixed, each haemoglobin is also located on the bell shaped curve of the Hill constant n versus log L. As seen in Fig. 2*B*, the reported values for n for each haemoglobin come very close to the calculated line. The data for *p*H 7 and *p*H 9 lie near the maximum of the curve accounting for the invariance of cooperativity accompanying affinity changes in the Bohr effect. Presumably the value of L changes in these regions because of the changes in the differential binding of protons by the R and T states (ref. 22 gives a more detailed explanation). The value for stripped haemoglobin also coincides approximately with the predicted curve, although the exact degree of cooperativity is not certain because some differences exist in the measured values[15,18]. The most interesting results, however, involve the mutant haemoglobins Chesapeake and Kansas. These two species represent a symmetric case of alterations in the α_1–β_2 interface of haemoglobin. In haemoglobin Chesapeake the change (leu FG4(92) $\alpha\rightarrow$arg) results in enhanced stability of the liganded tetramer as evidenced by diminished dissociation of oxyhaemoglobin (ref. 22 and R. Nagel, Q. H. Gibson and S. J. E., in preparation). In contrast haemoglobin Kansas (asn G4(102)$\beta\rightarrow$tyr) tends to dissociate more readily in the liganded form[19]. It is possible to demonstrate that the displacement of L for these mutants (Fig. 2*B*) corresponds quite well with the changes in the subunit dissociation, according to linkage principles (S. J. E., in preparation). Most significant, however, is the fact that both increases and decreases in stabilization diminish cooperativity to about $n=1.3$ in the two cases. This property is in striking agreement with the bell shaped dependence of cooperativity on L as shown in Fig. 2 and as predicted by a model based on conformational equilibrium. As will now be shown the sequential or induced fit model leads to a contrary prediction for these two mutants and poorly accommodates the Bohr effect.

Just as the value of L in the two state model is uniquely determined by the ligand binding affinity, so, too, is the principal parameter of the sequential model, K_{BB} in the nomenclature of Koshland *et al.*[4]. With the sequential model K_{BB} relates to the change in subunit bonding induced by the binding of ligand. The term K_{BB} is defined with respect to a single subunit interface, so that as many as six binding surfaces could be involved in a molecule such as haemoglobin, with pseudo-tetrahedral symmetry. As can be shown from linkage principles (S. J. E., in preparation), the dependence of K_{BB} on ligand binding affinity relative to isolated chains for haemoglobin gives a linear relationship of log $\alpha_{\frac{1}{2}}$ (or the ligand concentration at half saturation) versus log $(K_{BB})^6$. This relationship is identical to the dependence shown in Fig. 2*A*. In other terms $(K_{BB})^6$ is equivalent to L in that both are fixed according to linkage principles by the affinity of haemoglobin compared with its isolated chains: $L=(\alpha_{\frac{1}{2}})^4$ and $K_{BB}{}^6=(\alpha_{\frac{1}{2}})^4$.

With K_{BB} defined for the set of haemoglobins considered in Fig. 2, the question of cooperativity may now be considered. With the two state model, only c is freely adjustable to establish the proper cooperativity and a value of $c=0.01$ was

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selected to fit the behaviour at *p*H 7.0. This value then represented the other members of the haemoglobin set in conjunction with the values of L fixed by their respective affinities. For the sequential model the parameter K_{AB} is free to adjust to the degree of cooperativity. The term K_{AB} relates to the hybrid subunit bonding. In the sequential scheme, each fractionally saturated intermediate contains subunits in different conformations (liganded and unliganded) and K_{AB} relates to their interactions. In contrast, K_{BB} is dependent oniy on the relative bonding of a pair of liganded subunits compared with a pair of unliganded subunits. Because K_{AB} defines the strength and hence the relative occurrence of intermediates, the larger K_{AB}, the more intermediates and the lower the cooperativity. Conversely, as K_{AB} goes to zero, cooperativity or the Hill constant n tends to the maximum defined by the number of ligand binding sites. For haemoglobin at *p*H 7 the affinity corresponds to a value of $K_{BB}=1/9$ and the cooperativity corresponds to a value of $K_{AB}=2/3$. The change in cooperativity with change in affinity is given in Fig. 2*B* by the dashed line. The values for the sequential model were selected to coincide with the two state model near *p*H 7.0. But the sequential model, unlike the two state model, predicts a continuous increase in cooperativity with rising α_i (or log L which in turn is equivalent to log K_{BB}[6]). The behaviour of haemoglobin Kansas therefore falls far off the dashed curve. Moreover, the sequential model predicts a much steeper dependence of the changes in cooperativity with changing affinity than predicted by the two state model. The values of the Hill constant for stripped haemoglobins, haemoglobin at *p*H 9 and haemoglobin Chesapeake are thus much more poorly represented by the sequential model than with the two state model. The sequential model can fit the Hill constant for each haemoglobin in the set, but only by making arbitrary changes in the term K_{AB}. Its value must be lowered for haemoglobin Chesapeake and stripped haemoglobin, markedly raised for haemoglobin Kansas and altered in such a way as to just compensate for the invariance of cooperativity in the Bohr effect. A physical basis for such a pattern of changes is difficult to imagine. Thus, in terms of their ability to represent the ensemble of haemoglobin data, the two state model is very successful, whereas the sequential model requires a number of arbitrary adjustments.

Value and Limitations of Numerical Models

The molecular action of haemoglobin is complex and a battery of physical measurements will be required to unfold the intricate details. Dynamic techniques such as rapid reaction kinetics[16] have already provided much important information and in conjunction with the X-ray structures hold out the hope of a complete understanding at some future time. Eventually, they should provide more direct evidence about the applicability of the two state, sequential or some other model for haemoglobin. But it is worth emphasizing the value of some simple numerical exercises as described here. Even the more complex physical studies require values for the principal parameters of the competing models to orient the analysis of their data and a new method has been presented for obtaining these values in a more rigorous way than previously available. One result of the new method is to raise the value of the allosteric constant, L, for haemoglobin, from 10^4 to 3×10^5. The higher value should require a reconsideration of some of the experimental studies which have claimed to dismiss the presence of a conformational equilibrium[8]. Furthermore, a lower value of L (about 10^3) is now indicated by the relationship $L=(\alpha_i)^4$ for the spin labelled haemoglobin of Ogawa and McConnell[5]; the new value of L predicts that the R state and binding functions should be effectively coincident, as is in fact observed. The numerical exercises presented here are especially striking in terms of the haemoglobin data for the Bohr effect and mutants with high and low affinity (Fig. 2). The behaviour for all these cases is predicted by the two state model, once values of the constants are established for the "standard" data at *p*H 7.0. In marked contrast the values of the constants for the sequential model when rigorously set for *p*H 7.0 fail to accommodate the other haemoglobin data. Only by ancillary adjustments can all the data be accommodated. These comparisons cannot rule out the sequential model, but seriously weaken its validity.

In any consideration of models, it should be kept in mind that in many respects both the two state and sequential treatments must be gross simplifications. They can, of course, distinguish fundamental processes, such as the presence and absence of conformational equilibria. But, beyond this stage, there is an enormous body of detail concerning, for example, the exact kinetic rate for each process[15]. A knowledge of the structural basis of functional parameters such as L will also eventually be required. In this respect some information can be deduced from the subunit association–dissociation equilibria of native haemoglobin and its mutants. Preliminary examination indicates a close parallel between changes in these subunit equilibria measured in the analytical ultracentrifuge[23] and predicted value of L (Fig. 2*B*) when correlated by linkage principles[14].

In conclusion, this presentation emphasizes the likely presence of conformational equilibria. Whether or not only two states are involved in this equilibria is beyond the scope of this simple analysis. Conceivably an additional state, such as the half R–half T intermediate might be present, with marked implications for details of the kinetic or spectroscopic studies. Even with the addition of this state, model calculations show that results broadly the same as seen in Fig. 2 will be obtained. Therefore, an analysis of overall affinity and cooperativity properties can tend to exclude certain types of models such as the induced fit sequential treatment and favour conformational equilibria, but little can be said concerning the precise mechanism whereby the equilibria are expressed. Rather, information from dynamic experiments (see, for example, ref. 15) must provide these details.

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