

Linked Functions in Allosteric Proteins
Extension of the Concerted (MWC) Model for
Ligand-linked Subunit Assembly and its
Application to Human Hemoglobins

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The allosteric model of Monod *et al.* (1965) (MWC) has been extended to take into account the effects of subunit dissociation. The problem is formulated theoretically in terms of a general model for two allosteric species (dimers and tetramers) linked by a polymerization reaction. Relationships are presented for interpreting the dimer-tetramer association constants in terms of allosteric model parameters.

Sub-cases of the general model were tested against recent experimental data on the oxygenation-linked dimer-tetramer equilibria in normal human hemoglobin and in the variant hemoglobin Kansas (β_{102} , Asp \rightarrow Thr). The objectives of these analyses were: (1) to find the simplest models capable of describing the linked dimer-tetramer equilibria in the two hemoglobin systems, and (2) to evaluate the corresponding model parameters so that allosteric properties of the two hemoglobins may be compared.

In the simplest version of the model, the dimer is half of an R-state tetramer. This model was found to be excluded unequivocally by the data for both normal hemoglobin and hemoglobin Kansas when the α and β chains have equal binding affinities. When this two-state model was modified to permit non-equivalent affinities for the chains, the model could be fitted to hemoglobin Kansas, but not to hemoglobin A. A model, in which the dimers are allowed to exist in a state different from the tetramer R state, was found to be consistent with the data for hemoglobin A, with equivalent binding by the α and β chains. For hemoglobin A, the unliganded R-state tetramers have a different subunit dissociation energy from that of fully liganded R-state tetramers. The simplest model capable of describing both hemoglobin A and hemoglobin Kansas was obtained by extending this three-

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state model to permit (but not require) functional non-equivalence of the α and β chains. For these MWC models, unique estimates were obtained for the model parameters.

The allosteric constants for tetrameric hemoglobins A and Kansas are approximately equal. The value obtained from hemoglobin A is similar to previous estimates, whereas the value for hemoglobin Kansas is lower than previously estimated (Edelstein, 1971) by approximately two orders of magnitude. The low affinity of hemoglobin Kansas tetramer does not arise from an unusually high allosteric constant favoring the T-state species. It is largely the consequence of a greatly reduced oxygen affinity of β chains in the T state, and reduced values for the ratio between affinities in the R and T states.

1. Introduction

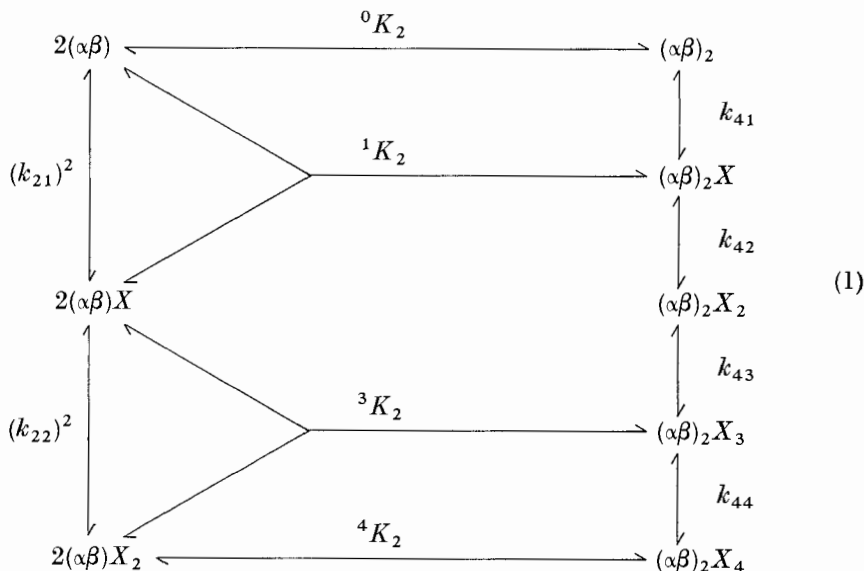
Multisubunit proteins such as human hemoglobin frequently exhibit cooperativity, which arises from a coupling between the effects of ligand binding at the individual subunits and the interactions between subunits of the assembled quaternary structure. A useful method of studying these coupling processes involves reversible dissociation of the molecule into its constituent protomeric units. The dissociation reactions eliminate the intersubunit contacts and thus provide a useful means of studying their role in the overall coupling process (Schejter *et al.*, 1963; Noble, 1969; Ackers & Halvorson, 1974; Colosimo *et al.*, 1976). Characterization of ligand-binding properties for the subunits in various stages of assembly, and of the dissociation reactions themselves, provides an experimental approach to understanding how assembly of the subunit chains into the functional oligomer brings about alterations in affinity and leads to the observed regulatory properties. A complete understanding of co-operative mechanisms will ultimately require the understanding of these processes in both structural and energetic terms. An important role of models lies in bridging the gap between the structural and energetic observations.

One of the simplest and most popular models for allosteric proteins is the two-state (concerted) model of Monod *et al.* (1965) (MWC). This model has been widely used as a first-order approximation to the behavior of human hemoglobins and other allosteric proteins. It provides one of the simplest descriptions of how co-operative ligand binding in an oligomeric protein may be linked to an equilibrium between two quaternary structures. In the case of human hemoglobins, the existence of at least two quaternary structures has been firmly established whereas their energetic properties are not very well understood. It is of interest to examine whether the subunit assembly of such an oligomeric protein can be described in all degrees of ligation by a two-state model. In this paper, we have extended the MWC theory (Monod *et al.*, 1965) to take into account the properties of dimers in equilibrium with the tetrameric hemoglobin molecules. We have also considered the effects of functional non-equivalence in the α and β chains on the extended model. The resulting models have been subjected to rigorous testing by numerical analysis against an extensive set of experimental data pertaining to both the normal human hemoglobin and the variant hemoglobin Kansas. Previous treatments of this problem (Edelstein, 1975; Thomas & Edelstein, 1973; Colosimo

et al., 1976) pertain only to a restricted sub-case of the model developed here. As will be shown, a more general model is necessary to account for the properties of both normal human hemoglobin and hemoglobin Kansas.

2. The Experimental System

A series of detailed experimental studies (Mills *et al.*, 1976; Johnson *et al.*, 1976; Ip *et al.*, 1976; Ip & Ackers, 1977; Valdes *et al.*, 1978; Mills & Ackers, 1979*a,b*; Atha & Riggs, 1976; Atha *et al.*, 1979) have recently provided a thermodynamic characterization for both normal hemoglobin and hemoglobin Kansas in terms of the reactions in the linkage scheme:



In this scheme, the tetrameric species are depicted as dimers of the $(\alpha^1\beta^1)$ units with which they are in reversible equilibrium[†]. Dissociation of the tetramers into these dimers eliminates the $\alpha^1\beta^2$ contact region where the major structural changes occur upon oxygenation (Perutz, 1976)[‡]. Estimates of the subunit association constants 0K_2 , 1K_2 , 3K_2 and 4K_2 are of special interest since variation in these quantities reflects the changes in intersubunit contact energy accompanying binding of the first, the middle two, and the last oxygen molecules. Using a model-independent thermodynamic analysis (Ackers & Halvorson, 1974), the constituent free energies, enthalpies and entropies of the reaction steps of the linkage scheme have been determined for the major component (A_0) of normal hemoglobin (Mills *et al.*, 1976; Johnson *et al.*, 1976; Ip *et al.*, 1976; Ip & Ackers, 1977; Valdes *et al.*, 1978; Mills & Ackers, 1979*a*). For hemoglobin Kansas, the free energies of the linkage scheme

[†] For a definition of constants, see the Appendix.

[‡] The hemoglobin tetramer $\alpha_2\beta_2$ had 2 types of contacts between dissimilar subunits denoted $\alpha^1\beta^1$ (equivalent to $\alpha^2\beta^2$) and $\alpha^1\beta^2$, respectively (Perutz, 1976).

have been determined at a single temperature under comparable conditions to those used when the hemoglobin A results were obtained (Atha & Riggs, 1976; Atha *et al.*, 1979)†. A summary of the free energies for hemoglobins A and Kansas is presented in Table 1. These basic thermodynamic quantities impose constraints that must be met by all models of hemoglobin action. It is thus possible to rule out models whenever inconsistencies are found, although consistency does not provide positive verification of a model's correctness.

TABLE 1

Model-independent thermodynamic properties for the linkage system in hemoglobins A and Kansas

Free energy	Hemoglobin A†	Hemoglobin Kansas‡
ΔG_{21} §	- 8.38 ± 0.2	- 8.40
ΔG_{22} §	- 8.38 ± 0.2	- 7.34
ΔG_{41}	- 5.45 ± 0.2	- 5.81 ± 0.1
ΔG_{42}	- 5.28 ± 0.5	- 5.41 ± 0.4
ΔG_{43}	- 7.80 ± 0.6	- 5.59 ± 0.5
ΔG_{44}	- 8.65 ± 0.4	- 6.36 ± 0.4
${}^0\Delta G_2$ ¶	- 14.38 ± 0.2	- 13.63 ± 0.2
${}^1\Delta G_2$ ¶	- 11.46 ± 0.3	- 11.41 ± 0.2
${}^3\Delta G_2$ ¶	- 7.78 ± 0.2	- 7.48 ± 0.2
${}^4\Delta G_2$ ¶	- 8.05 ± 0.2	- 5.29 ± 0.2

† Data analyzed are those of Mills *et al.* (1976) and Ip & Ackers (1977), conditions: 0.1 M-Tris·HCl, pH 7.4, 0.1 M-NaCl, 1 mM-Na₂EDTA, 21.5°C. Values are taken from Mills *et al.* (1976); variance of fit = 2.57×10^{-5} .

‡ Data of Atha *et al.* (1979); conditions: 0.05 M-Tris·HCl, pH 7.5, 0.1 M-NaCl, 1 mM-Na₂EDTA, 20.0°C; variance of fit = 1.3×10^{-4} .

§ Free energies for sequential binding reactions of oxygen by dimers, corrected for statistical factors.

|| Free energies for sequential binding reactions of oxygen by tetramers, corrected for statistical factors.

¶ Free energy of formation of tetramers ${}^i\Delta G_2$ with i oxygens bound, corrected for statistical factors.

In molecular terms, the observed changes in dimer-tetramer association energy ${}^i\Delta G_2$ that accompany the changes in ligation state (Table 1) could be accounted for by either or both of two opposing concepts: (1) purely "sequential" models require the changes in ${}^i\Delta G_2$ with the successive oxygenation steps to reflect changes in energy within a single tetrameric molecule as compared to the corresponding changes in a single dimeric molecule. (2) Purely "concerted" models, such as those considered in this paper, permit no "communication" between subunits of an individual molecule. The values of ${}^i\Delta G_2$ therefore represent averages of values that

† Experimental information used in the thermodynamic analyses has included (1) kinetic determinations of forward and reverse reactions for association of unliganded hemoglobin, yielding values of the equilibrium constant 0K_2 , (2) analytical gel chromatography data, yielding values of 4K_2 , and (3) oxygenation curves measured as a function of hemoglobin concentration (i.e. between 4×10^{-8} M-heme and 4×10^{-4} M-heme for hemoglobin A and between 4×10^{-7} M-heme and 6×10^{-3} M-heme for hemoglobin Kansas), from which in combination with (1) and (2) the remaining parameters were determined. For hemoglobin A, determination of these constants as a function of temperature has permitted the estimation of van't Hoff enthalpies and entropies for the microscopic reaction steps (Mills & Ackers, 1979).

pertain to different conformational isomers of the dimers and tetramers in equilibrium. These conformational isomers occur in a "pre-existing" equilibrium and the relative distribution of species is shifted by ligand binding as a result of differences in ligand affinity between the conformational states.

3. Theory

Our main objective in this section is to show how the basic thermodynamic parameters of the dimer-tetramer linkage scheme (1) can be "translated" into the formalism of the MWC model at several relevant levels of generality. The resulting models differ in the assumptions made regarding properties of the dimeric species, and regarding the equivalence in binding affinities of α and β chains.

(a) General assumptions: the MWC model

The original model of Monod *et al.* (1965) has been discussed in a number of reviews in terms of its relation to hemoglobin (Edelstein, 1975; Schulman *et al.*, 1975; Baldwin, 1975). The discussion here will be limited to a brief synopsis of the principal concepts underlying the theory, as required for the extensions of the model developed in this work.

The underlying concept of the MWC model is that of an equilibrium between two quaternary structures (denoted R and T), which differ in their intrinsic affinities for ligands. Transitions between the two states are assumed to be "concerted" in the sense that all affinities of binding sites in a given molecule change in concert with the T \rightarrow R transition. Furthermore, the equilibrium between R and T states is assumed to exist in the absence of bound ligand and thus to be a property of the protein alone. The observed co-operativity arises from the linkage between this concerted conformational equilibrium and the binding reactions of the two forms that differ in affinity. A conspicuous feature of the model is that it does not permit any communication within a given molecule, i.e. binding of the first ligand to a T-state tetramer does not alter the affinities of any other sites within the same molecule. The coupling between binding sites and intersubunit contact sites is entirely an indirect effect of mass action.

The equilibrium between R and T states at any state of ligation, i , is defined by the allosteric constant iL_n :

$${}^iL_n = \frac{[(\alpha\beta)_{n/2}^T X_i]}{[(\alpha\beta)_{n/2}^R X_i]}, \quad i = 0, 1, \dots, n \quad (2)$$

$$n = 2, 4$$

where $[(\alpha\beta)_{n/2}^T X_i]$ and $[(\alpha\beta)_{n/2}^R X_i]$ are the concentrations of T-state and R-state oligomers containing n subunit sites and with i ligands bound. The allosteric constants satisfy the relationship ${}^iL_n = {}^0L_n c_n^i$, where c_n is the ratio of ligand association constants for T-state molecules, K_T , and R-state molecules, K_R ; i.e. $c_n = K_T/K_R$. The Adair binding constants K_{ni} , which are products of the sequential constants (denoted in scheme (1) by lower case k) are related to the MWC parameters (for the case of equivalent subunit bindings sites) through the relations:

$$K_{ni} = \binom{n}{i} \frac{(1 + {}^0L_n c_n)}{(1 + {}^0L_n)} \cdot (K_R)^i, \quad (3)$$

where $\binom{n}{i}$ is the binomial coefficient representing the standard statistical factor for multisite ligand binding. Relations equivalent to equation (3) have been discussed by Imai (1973), Thomas & Edelstein (1973) and Edelstein (1975). With these binding constants, the standard binding isotherm:

$$\bar{Y}_n = \frac{1}{n} \frac{\sum_{i=0}^n i K_{ni} [X]^i}{\sum_{i=0}^n K_{ni} [X]^i}; \quad K_{n0} = 1 \quad (4)$$

is transformed (Monod *et al.*, 1965) into:

$$\bar{Y}_n = \frac{{}^0L_n c_n \alpha_n (1 + c_n \alpha_n)^{n-1} + \alpha_n (1 + \alpha_n)^{n-1}}{{}^0L_n (1 + c_n \alpha_n)^n + (1 + \alpha_n)^n}, \quad (5)$$

where $\alpha_n = [X]K_R$. For an oligomer with n equivalent binding sites that conforms to an MWC mechanism, the number of constants required to define the binding isotherm is changed from n to three (i.e. 0L_n , c_n and K_R).

(b) *Extension of the MWC model for the linked dimer-tetramer system with equivalence of chains*

The most general application of the MWC concepts summarized above to the linkage scheme (1) would be to assume R and T states for the dimer as well as the tetramer so that relations of the type given by equations (2), (3) and (5) could be written to describe the allosteric equilibria of dimeric species as well. In general, then, a four-state model would account for properties of the linkage system. In this section, we present the formulation of this general allosteric model. The corresponding formulation of this linkage problem in terms of the model-independent thermodynamic constants has been given by Ackers & Halvorson (1974).

(i) *The binding isotherm for the linked system*

The theoretical binding isotherm for the dimer-tetramer association reaction linked with oxygen binding, as observed in human hemoglobin, is given by:

$$Y_{2,4} = \frac{Z_2 + Z'_4 (\sqrt{Z_2^2 + 4{}^0K_2 Z_4 [P_t]} - Z_2) / 4Z_4}{Z_2 + \sqrt{Z_2^2 + 4{}^0K_2 Z_4 [P_t]}}, \quad (6)$$

where

$$Z_2 = 1 + K_{21}[X] + K_{22}[X]^2; \quad Z'_2 = K_{21}[X] + 2K_{22}[X]^2 \quad (7a,b)$$

$$Z_4 = 1 + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4 \quad (8a)$$

$$Z'_4 = K_{41}[X] + 2K_{42}[X]^2 + 3K_{43}[X]^3 + 4K_{44}[X]^4, \quad (8b)$$

where $[P_i]$ is the total protein concentration in molar heme, 0K_2 is the subunit association constant to form unliganded tetramers from unliganded dimers, and $[X]$ is the molar oxygen concentration. K_{2i} and K_{4i} are the product Adair constants for dimeric and tetrameric species, respectively. Equations (6) to (8b) provide an algorithm for formulating the binding isotherm $Y_{2,4}$ in terms of any model for which values of the Adair constants K_{ni} can be specified as functions of the model parameters. For the MWC model with equivalent binding sites this function is provided by equation (3). For the case of non-equivalent binding by the α and β chains the corresponding formula is presented in section (iii), below. The function $Y_{2,4}$ defined by equations (3) and (6) to (8b) provides a formulation of the general thermodynamic isotherm of Ackers & Halvorson (1974) in terms of the seven constants (0K_2 , 0L_4 , c_4 , K_{R4} , 0L_2 , c_2 , K_{R2}). It may be noted that the standard thermodynamic formulation (equation (6)) also requires seven constants (0K_2 , K_{21} , K_{22} , K_{41} , K_{42} , K_{43} , K_{44}). Thus, the MWC formulation does not provide any reduction in the number of parameters required to describe the linkage system in the general case, even when no distinction exists between affinities of α and β chains. When a distinction does exist, the number of parameters required is increased to 11, as will be seen in section (iii), below.

(ii) *Subunit association constants for the linked system*

An alternative linkage function, which has been used to characterize scheme (1) (Ackers & Halvorson, 1974; Valdes *et al.*, 1978), is the dimer-tetramer equilibrium constant xK_2 :

$${}^xK_2 = {}^0K_2 \frac{Z_4}{(Z_2)^2}. \quad (9)$$

The linkage function xK_2 is an average, which depends upon the oxygen concentration, $[X]$, and the seven independent constants of the linkage scheme which define Z_4 , Z_2 , and 0K_2 . Substitution of the relations given in equation (3) for the constants K_{ni} leads to:

$${}^xK_2 = {}^0K_2 \frac{{}^0L_4 (1 + c_4 \alpha_4)^4 + (1 + \alpha_4)^4}{[{}^0L_2 (1 + c_2 \alpha_2)^2 + (1 + \alpha_2)^2]^2}. \quad (10)$$

In considering the relationship of the linkage scheme (1) to allosteric models, it is of interest to formulate an interpretation in terms of model parameters of the experimentally determinable dimer-tetramer association constants iK_2 corresponding to ligation states $i = 0, 1, 3, 4$. The only treatments of this problem in the literature (Edelstein, 1975; Thomas & Edelstein, 1973; Colosimo *et al.*, 1976) pertain to a restricted sub-case of the general model which, as will be shown, does not fit the recent data for either of the hemoglobins investigated.

The following derivation assumes that each species (dimers or tetramers) has a separate allosteric equilibrium between R and T states and that these four conformational states may all be different. The experimentally measured association equilibrium constant for unliganded hemoglobin is then given by:

$${}^0K_2 = \frac{[(\alpha_2\beta_2)^R] + [(\alpha_2\beta_2)^T]}{[(\alpha\beta)^R] + [(\alpha\beta)^T]^2} = {}^0K_{2R} \frac{(1 + {}^0L_4)}{(1 + {}^0L_2)^2}, \quad (11)$$

where brackets denote concentrations of species contained within them, superscripts R and T denoted states of the dimers ($\alpha\beta$) and tetramers ($\alpha_2\beta_2$) (note again that the T and R states of dimers may be functionally different from those of tetramers). ${}^0K_{2R}$ is the equilibrium constant for forming R-state tetramers, defined by:

$${}^0K_{2R} = \frac{[(\alpha_2\beta_2)^R]}{[(\alpha\beta)^R]^2}. \quad (12)$$

Similarly, for formation of tetramers at the other states of ligation: 1, 3 and 4, we have:

$${}^1K_2 = {}^1K_{2R} \frac{(1 + {}^0L_4c_4)}{(1 + {}^0L_2)(1 + {}^0L_2c_2)}; \quad {}^1K_{2R} = \frac{[(\alpha_2\beta_2)^R X]}{[(\alpha\beta)^R][(\alpha\beta)^R X]} \quad (13a, b)$$

$${}^3K_2 = {}^3K_{2R} \frac{(1 + {}^0L_4c_4^3)}{(1 + {}^0L_2c_2)(1 + {}^0L_2c_2^2)}; \quad {}^3K_{2R} = \frac{[(\alpha_2\beta_2)^R X_3]}{[(\alpha\beta)^R X][(\alpha\beta)^R X_2]} \quad (14a, b)$$

and

$${}^4K_2 = {}^4K_{2R} \frac{(1 + {}^0L_4c_4^4)}{(1 + {}^0L_2c_2^2)^2}; \quad {}^4K_{2R} = \frac{[(\alpha_2\beta_2)^R X_4]}{[(\alpha\beta)^R X_2]^2}. \quad (15a, b)$$

These formulae define each of the experimentally accessible dimer-tetramer constants in terms of the allosteric model parameters and the dimer-tetramer association constants pertaining to the R-state tetramers ${}^iK_{2R}$. It should be noted that these constants may be different from each other. In the most general cases, the only further reduction possible comes from noting that they are related to a set of intrinsic constants ${}^iK'_{2R}$ through statistical factors: ${}^iK_{2R} = s_i {}^iK'_{2R}$, where $s_i = 1, 2, 2, 1$ for values of $i = 0, 1, 3, 4$, respectively. These statistical factors account for the various microscopic configurations of the species involved in the equilibrium between subunits in the same way that statistical factors relate macroscopic ligand-binding constants to the intrinsic constants. The intrinsic dimer-tetramer association constant, ${}^iK'_{2R}$, then reflects the energy of forming the appropriate molecular complex independent of the number of ways of doing it.

The relationships between the dimer-tetramer constants discussed above and the ligand-binding constants of the linkage scheme may be seen by noting that conservation of energy requires:

$$k_{21} = \frac{{}^0K_2}{{}^1K_2} k_{41} \quad (16)$$

$$k_{22} = \frac{{}^3K_2}{{}^4K_2} k_{44}. \quad (17)$$

Then substituting from equation (3) and the relationships discussed above for the iK_2 terms, we have:

$$k_{21} = 2K'_R \frac{(1 + {}^0L_2 c_2)}{(1 + {}^0L_2)} \cdot \frac{{}^0K'_{2R}}{{}^1K'_{2R}}; \quad k_{22} = \frac{K'_R}{2} \frac{(1 + {}^0L_2)}{(1 + {}^0L_2 c_2^2)} \cdot \frac{{}^3K'_{2R}}{{}^1K'_{2R}}, \quad (18a, b)$$

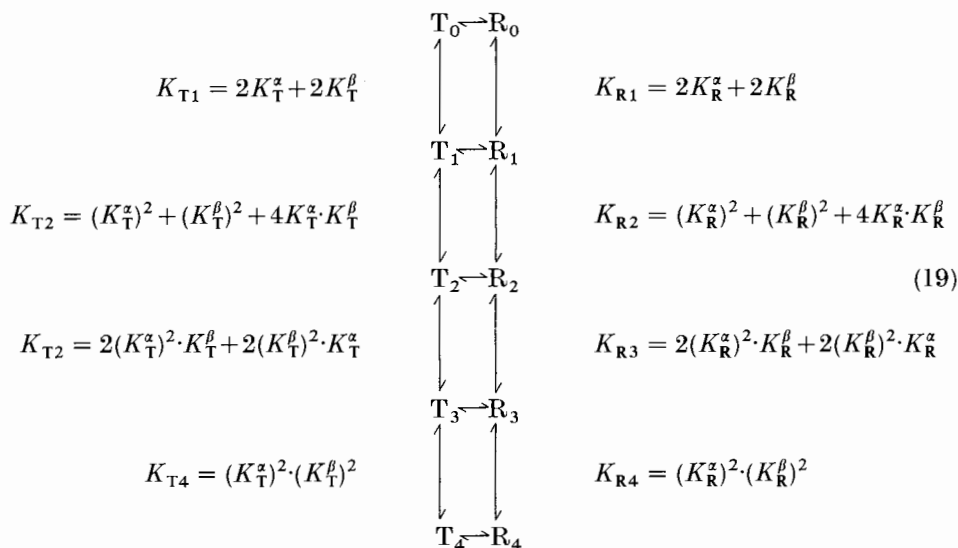
where K'_R is the intrinsic binding constant to R-state tetramers.

The relationships summarized in the above two sections provide a complete formulation of the extended MWC model for any linked dimer-tetramer system in which the chains bind equivalently. For a given experimental system, certain simplifications or further extensions of this model may be found desirable. At this point, it should be noted that a model of this general type could, in principle, account for a higher overall affinity for dimers as compared with tetramers either through a smaller value for the dimer allosteric constant, i.e. ${}^0L_2 < {}^0L_4$ (so that the allosteric equilibria of dimers lie further in the direction of the R state than do those of tetramers) or through higher intrinsic binding constants (K_R, K_T) for the dimers.

(iii) *Functional non-equivalence of chains*

In hemoglobin Kansas the isolated α and β chains have significantly different affinities for oxygen (Riggs & Gibson, 1973) and the analysis of recent oxygenation data for hemoglobin Kansas indicates that these differences are also present in dimers and tetramers (Atha *et al.*, 1979). It is therefore of interest to consider the extensions of the general linkage model. An extended model would also be applicable to hemoglobin A in the presence of organic phosphate where the subunits have different affinities (Johnson & Ho, 1974).

Formulae for binding isotherms of MWC tetramers with non-equivalent chains have been presented by Ogata & McConnell (1971) and Edelstein (1974). The present analysis may be viewed as a generalization of their treatments to a linked polymerizing system. As noted earlier, the formulation of any model for this linked system may be carried out simply by "translating" the Adair constants, K_m , for dimers and tetramers from an appropriate set of parameters derived from the model of interest. Then equations (6) to (8b) complete the formulation. If the α and β chains are not equivalent, there are four microscopic binding constants for each of the oligomeric species. For the α chains we have K_R^α and K_T^α , which describe binding to those chains when they exist in the R and T-state quaternary structures. The corresponding constants for β chains will be K_R^β and K_T^β , respectively. The binding constants for R-state and T-state molecules will involve combinations of these four microscopic constants. For tetramers these combinations are shown in the scheme below.



The binding constants $K_{\text{R}i}$ and $K_{\text{T}i}$ describe the binding of i ligands onto an R-state or T-state tetramer, respectively; e.g.

$$K_{\text{R}3} = \frac{[(\alpha_2\beta_2)^{\text{R}}X_3]}{[(\alpha_2\beta_2)^{\text{R}}][X]^3}. \tag{20}$$

The Adair binding constants each representing the binding of i ligands onto tetramers of all forms are, therefore:

$$\begin{aligned}
 K_{4i} &= \frac{[(\alpha_2\beta_2)X_i]}{[(\alpha_2\beta_2)][X]^i} = \frac{[(\alpha_2\beta_2)^{\text{T}}X_i] + [(\alpha_2\beta_2)^{\text{R}}X_i]}{[(\alpha_2\beta_2)^{\text{T}} + (\alpha_2\beta_2)^{\text{R}}][X]^i} \\
 &= \frac{1 + c_i {}^0L_4}{1 + {}^0L_4} K_{\text{R}i}; \quad i = 1, 2, 3, 4,
 \end{aligned} \tag{21}$$

where

$$c_i = \frac{K_{\text{T}i}}{K_{\text{R}i}}.$$

A similar analysis for the dimers yields:

$$\begin{aligned}
 K_{\text{R}1} &= K_{\text{R}}^\alpha + K_{\text{R}}^\beta; & K_{\text{R}2} &= K_{\text{R}}^\alpha \cdot K_{\text{R}}^\beta \\
 K_{\text{T}1} &= K_{\text{T}}^\alpha + K_{\text{T}}^\beta; & K_{\text{T}2} &= K_{\text{T}}^\alpha \cdot K_{\text{T}}^\beta
 \end{aligned} \tag{22}$$

and the Adair constants are:

$$K_{2i} = \frac{1 + c_i {}^0L_2}{1 + {}^0L_2} K_{\text{R}i}; \quad i = 1, 2. \tag{23}$$

When these product Adair constants given by equations (21) and (23) are substituted into the binding isotherms (equations (6) to (8b)), we have a complete

definition of the binding isotherm for the linked system in terms of this general model. It may be noted that this model in its most general form requires 11 constants, i.e. the eight microscopic constants K_R^α , K_R^β , K_T^α , K_T^β (four of these for dimers and another four for tetramers) plus 0L_2 , 0L_4 and 0K_2 .

4. Methods

(a) Computational approach

We have developed a computational program that carries out non-linear least-squares analysis of experimental data in terms of parameters from any particular model that is specified (for example, the L , c and K_R constants of the MWC model). This program utilizes a modification of the Gauss-Newton iterative technique that had been developed previously for analysis of data in terms of the equilibrium constants of the linkage scheme (equation (1)) (Johnson *et al.*, 1976). A major modification of the program described previously was required by the complexity of evaluating the required partial derivatives of the saturation function with respect to each of the model parameters. In the present program, all of the derivatives required for the model fitting are evaluated by numerical differentiation using a 5-point Lagrangian interpolation formula (Hildebrand, 1956).

A subroutine is written in the case of each model, which translates the model parameters into the 7 thermodynamic constants, 0K_2 , K_{21} , K_{22} , K_{41} , K_{42} , K_{43} and K_{44} . Any of the model parameters may be held fixed, allowed to "float", or set equivalent to any of the other model parameters. The program then fits raw experimental data directly to the model parameters and generates confidence profiles and statistics in the same way as described earlier (Johnson *et al.*, 1976). The procedure for error estimates is basically a mapping of the variance space to find an F -statistic that corresponds to a 90% confidence interval. The maximum deviation of a parameter is then taken as its confidence interval.

In judging the goodness of fit for any particular model to the experimental data, we imposed essentially the same criteria as were used in obtaining the best fits to the basic thermodynamic parameters of the linkage system (Mills *et al.*, 1976; Johnson *et al.*, 1976; Mills & Ackers, 1979*a,b*; Atha *et al.*, 1979). The assessments were based upon (1) the value of the variance of the fit as compared with the corresponding variance of fit to the thermodynamic constants, (2) randomness in the distribution of residuals of the best-fit, (3) correlation between the fitted parameters, and (4) comparison between values of the thermodynamic constants calculated using the best-fit model parameters and the error limits for their values obtained from fitting the same data to the thermodynamic constants alone.

(b) Data analyzed

Experimental data analyzed in this study included:

(i) For hemoglobin A

(1) The concentration-dependent oxygenation data (Mills *et al.*, 1976; Mills & Ackers, 1979*a,b*) combined with independent results on dimer-tetramer association of unliganded and fully oxygenated hemoglobin (Ip & Ackers, 1977). These data were obtained at pH 7.4 in 0.1 M-Tris·HCl, 0.1 M-NaCl, 1 mM-Na₂EDTA, and were determined as a function of temperature over the range 10 to 37°C†. (2) For comparison, we have also analyzed the data of Roughton & Lyster (1965) for allosteric properties of tetramers.

† Under conditions pertaining to these data, the nuclear magnetic resonance results have established the affinities of α and β chains within tetrameric hemoglobin A to be identical to within approximately 5%.

(ii) *For hemoglobin Kansas*

We have analyzed the concentration-dependent oxygen binding data of Atha *et al.* (1979) combined with data of Atha & Riggs (1976) on the dimer-tetramer association constants. These data were obtained at pH 7.5 in 0.05 M-Tris·HCl, 0.1 M-NaCl, 1 mM-NA₂EDTA at 20°C.

5. Results

Our strategy in analyzing data for hemoglobins A and Kansas was to begin with the simplest models and to test them against the experimental data, proceeding in complexity only until the minimum model capable of describing the data was found.

(a) *Hemoglobin A*

In the case of normal hemoglobin A, the observed non-co-operativity of dimers (see Table 1) precludes an appreciable allosteric mechanism of the MWC type to be operative in those species, since this would require positive co-operativity in the binding of oxygen by dimers (Monod *et al.*, 1965). For this hemoglobin, we analyzed the data according to the sub-cases of the general model that retain the allosteric properties of equations (2) and (3) for tetramers, but allow the dimers to be non-co-operative. We considered two such models and present tests of their conformity to the experimental results.

(i) *The two-state model for the linked dimer-tetramer system*

The simplest MWC model for the linked dimer-tetramer system would include two allosteric states (R and T) for the tetramers and only a single state (R) for the dimers. A model of this type has been considered by Edelstein (1971,1975) and Colosimo *et al.* (1976) and follows from the original MWC assumption that the entire difference between R and T-state tetramers arises from interactions *between* the assembled protomers (dimers). Thus, the protomers are expected to exist in the R state in their dissociated form. We impose three constraints on the general model: (1) we assume no allosteric properties for dimers, so that ${}^0L_2 = 0$. (2) The dissociated dimers are assumed to have the same binding properties as R-state tetramers. (3) As necessary consequence of a strictly two-state model, the subunit association constant for R-state (or T-state) tetramers must be independent of the degree of ligation so that ${}^0K'_{2R} = {}^1K'_{2R} = {}^3K'_{2R} = {}^4K'_{2R}$. Denoting each of these constants by K_{2R} , the experimentally measured macroscopic dimer-tetramer association constants are:

$${}^iK_2 = s_i K'_{2R} (1 + {}^0L_4 c_4^i), \quad (24)$$

where s_i is the appropriate statistical factor for each ligation state. In this simplest extension of the MWC model, the assumptions described above imply non-co-operativity in ligand binding by the dissociated dimers, since from equations (18a, b):

$$k_{21} = 2K_R \quad (25)$$

$$k_{22} = K_R/2 \quad (26)$$

and consequently $k_{21}/k_{22} = 4$, the standard ratio of statistical factors for a binding system with two independent sites. A complete translation between this model and the thermodynamic constants of the linkage scheme (1) is provided by equations (3) and (24) to (26).

For purposes of testing the *two-state model* against experimental data we utilized the fact that k_{21} , k_{22} (hence K_R) and 0K_2 are determined in a model-independent fashion, and equation (3) defines the four remaining constants, K_{4i} , necessary to describe the linkage scheme completely. Therefore only 0L_4 and c_4 need be specified in order to fix the values of all parameters. This can be reduced to a single-parameter fitting problem by utilizing the relationship:

$${}^0K_2 = \frac{1 + {}^0L_4}{1 + {}^0L_4 c_4^4}, \quad (27)$$

which follows from equation (24), and noting that 4K_2 is also determined from the experimental data in a model-independent fashion. One of the remaining constants of the model (0L_4 or c_4) can be eliminated between equations (3) and (27). Thus in fitting to the data only a single parameter must be estimated in order to define the dimer-tetramer linkage scheme in terms of this model.

The least-squares fits we obtained for the models tested appeared to yield unique minima, which were independent of the initial guesses, so that unique parameter values were obtained. Results of the analyses for a typical data set, that of Mills *et al.* (1976) pertaining to 21.5°C, are shown in Figure 1 and Table 2. Figure 1 shows that the distribution of residuals to the best fit for the two-state model exhibited a pronounced skewing (Fig. 1(c)) as compared with the distribution obtained from model-independent thermodynamic fits (Fig. 1(b)). The two-state model also had a considerably higher variance of fit (7.4×10^{-5}) than that of the model-independent fit (2.5×10^{-5}).

A version of the two-state model was also tested in which functional non-equivalence of the α and β chains was allowed, but no variations in values of these parameters were found that would bring the variance of fit and distribution of residuals within correspondence to that of the thermodynamic fits (Mills *et al.*, 1976). The same behavior was also exhibited at each of the data sets at five

TABLE 2
Estimated model parameters for hemoglobin A at 21.5°C

Model parameter	Two-state† MWC	Three-state‡ MWC
0L_4	5.2×10^4	5.6×10^5 (3.0×10^5 , 9.2×10^5)
c	6.1×10^{-3} (5.4×10^{-3} , 6.8×10^{-3})	3.5×10^{-3} (3.0×10^{-3} , 3.9×10^{-3})
K_R	1.73×10^6	3.1×10^6 (2.7×10^6 , 3.5×10^6)
δ_{04}	0	1.39 kcal (1.0, 1.6)

† Parameters corresponding to best fit of data from model in which dimers are equivalent to half of R-state tetramers. Variance of fit = 7.4×10^{-5} .

‡ Parameters from fit to model in which dimers are allowed to have different energy states from R-state tetramers. Variance to fit = 2.6×10^{-5} . Values in parentheses are 90% confidence limits.

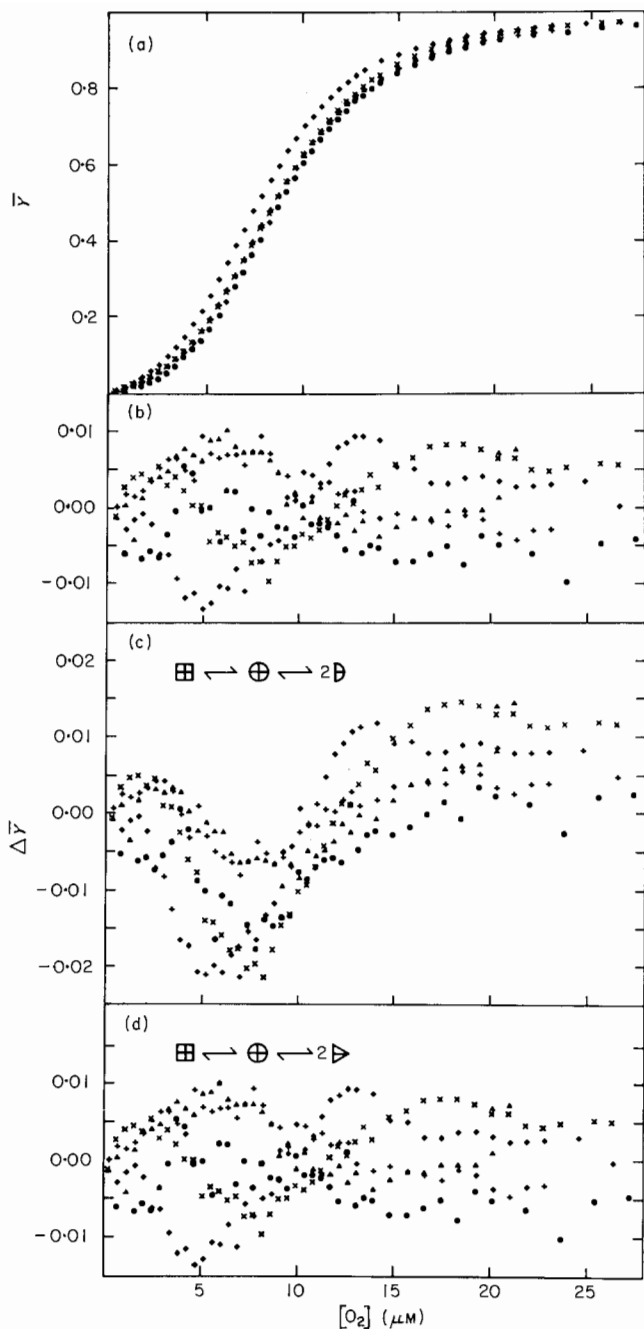


FIG. 1. Analysis of the concentration-dependent oxygenation data of Mills *et al.* (1976) for normal human hemoglobin (A_0). (a) The raw experimental data collected at a hemoglobin A_0 concentration between 5×10^{-6} M-heme and 4×10^{-4} M-heme. (b) The distribution of residuals for the best fit in the previously published thermodynamic analysis of Mills *et al.* (1976). (c) The distribution of residuals from the least-squares fit to a 2-state MWC mechanism where the dimers are equivalent to R-state tetramers. (d) Distribution of residuals from the least-squares fit to a 3-state MWC model where the properties of dimers are not identical to either R or T-state tetramers.

temperatures covering a range between 10 and 37°C. At none of the temperatures was the two-state model capable of describing the data for the linked dimer-tetramer system as well as the same data could be fitted to the model-independent thermodynamic constants. This result is not surprising in view of the recent findings of quaternary enhancement effects (Mills & Ackers, 1979*a,b*), which on model-independent grounds require the dimers to have a lower affinity for oxygen than that of triliganded tetramers.

(ii) *A three-state model for the linked dimer-tetramer system*

In the two-state model we have made three assumptions regarding the properties of dimers. The first assumption (1) and that of equivalent chains imply non-cooperativity of dimers as shown above. These assumptions do not require, however, that the intrinsic affinity of R-state tetramers be equal to K'_R . The second assumption (2) is equivalent to ${}^0K'_{2R} = {}^1K'_{2R} = {}^3K'_{2R} = {}^4K'_{2R}$ and requires that the assembly of dimers into R-state tetramers results in a quaternary structure with unaltered affinity. It is of interest to consider a more general version of the model in which assumption (2) is relaxed, permitting separate intrinsic binding energies for dimers and R-state tetramers. An alternative manifestation of this property, arising from the linkage relations, is that R-state tetramers dissociate with different energies depending upon their degree of ligation. The difference, δ_{04} , between free energies of forming R-state tetramers in unliganded and fully oxygenated states is:

$$\delta_{04} = -RT \ln \frac{{}^0K'_{2R}}{{}^4K'_{2R}}. \quad (28)$$

This energy also equals four times the difference between intrinsic binding energies for dimers and R-state tetramers, i.e.

$$\delta_{04} = -RT \ln [(k'_{21})^4/(K'_R)^4] = 4(\Delta G'_{21} - \Delta G'_R), \quad (29)$$

where $\Delta G'_R = -RT \ln K'_R$. When the two kinds of chains are functionally equivalent, the increment in quaternary enhancement or constraint that accompanies dimer-tetramer assembly is equal to $1/4(\delta_{04})$ for each successive ligation step.

For the *three-state model*, the relationship corresponding to equation (29) is:

$$\delta_{04} = RT \ln 4 \frac{{}^0K_2(1 + {}^0L_4c_4^4)}{{}^4K_2(1 + {}^0L_4)}. \quad (30)$$

In this case, the independent estimation of 0K_2 , 4K_2 , k_{21} and k_{22} permits the use of equations (3) and (30) to complete the linkage scheme by simultaneously finding the best values of any two of the quantities 0L_4 , c_4 or δ_{04} .

Results of fitting to the three-state model are shown in Figure 1(d), where the distribution of residuals is essentially identical to that of the model-independent analysis (Fig. 1(b)) and also the variance of fit (2.57×10^{-5}) was quite comparable. This behavior was found for each of the data sets at the different temperatures, so

that all of the hemoglobin A data are consistent with this model. The values of the free energies of ligand binding and subunit assembly derived from these fits to the two-state and three-state models are quite similar to those of the model-independent fit (Table 1). Comparison of these energies does not in this case provide as stringent a test of the models as the tests based on variance of fit and distribution of residuals. Table 2 lists the MWC parameters obtained in the fits along with their 90% confidence limits. It can be seen that the tetrameric allosteric constant, 0L_4 , requires less than one tetramer in 100,000 to be in the R state for deoxy hemoglobin A, and the affinities of the two tetrameric states differ by several hundred-fold, as indicated by the value of c . The intrinsic affinity, K_R , of the R-state tetramer is approximately twice as high in the three-state model as that of the dimers (in the two-state model the two are equal). Finally, it may be noted from the value of δ_{04} (Table 2) that the free energies of formation of R-state tetramers in their oxygenated and deoxygenated states differ by 1.4 kcal (see eqn (30)).

(iii) *Temperature dependence of the allosteric constants*

A summary of the temperature dependence of the MWC parameters (derived from fits to the three-state model) is given in Table 3. From a van't Hoff analysis of these results, the apparent enthalpies and entropies corresponding to 0L_4 , K_R and K_T were also obtained: $\Delta H_L = -30.6 \pm 11.2$ kcal, $\Delta H_R = -18.7 \pm 3.6$ kcal and $\Delta H_T = -9.5 \pm 2.0$ kcal, respectively. The enthalpy, ΔH_L , of the allosteric transition is considerably higher than that estimated by Gaud *et al.* (1975), from comparison between heats of ligation for the variant hemoglobin M Iwate (presumably always in the T-state) and hemoglobin A. The enthalpy is close to that estimated for trout I hemoglobin by Barisas & Gill (1979). The value we obtain in this study for ΔH_R is comparable to enthalpies of oxygen binding to dimers and for isolated α and β chains (Mills *et al.*, 1979; Mills & Ackers, 1979*a,b*).

The values of ΔH_T and ΔH_R are indistinguishable, to within error limits, from the model-independent enthalpies of binding for the first and last oxygenation steps, respectively (Mills & Ackers, 1979*b*). This is to be expected from the small fractional changes in position of the allosteric equilibria that accompany these ligation steps.

TABLE 3

Three-state MWC parameters for hemoglobin A as a function of temperature†

$t(^{\circ}\text{C})$	ΔG_L	ΔG_c	$1/4(\delta_{04})$	ΔG_R
10	-10.5 ± 0.5	3.8 ± 0.2	0.8 ± 0.2	-9.4 ± 0.2
15	-11.4 ± 0.5	4.1 ± 0.2	1.2 ± 0.2	-9.6 ± 0.2
21.5	-9.0 ± 0.4	3.6 ± 0.3	0.7 ± 0.3	-9.0 ± 0.4
33	-8.9 ± 1.1	3.5 ± 0.3	0.9 ± 0.4	-8.4 ± 0.2
37	-9.0 ± 0.4	3.5 ± 0.2	1.0 ± 0.2	-8.9 ± 0.2

† Free energies are obtained from $\Delta G = -RT \ln K$, where K represents the appropriate constant: ΔG_L corresponds to the allosteric constant 4L_2 , ΔG_c pertains to c , ΔG_R pertains to the intrinsic binding energy for R-state tetramers. $1/4(\delta_{04})$ is the difference in binding free energy between dimers and R-state tetramers, represented here in terms of one oxygen. δ_{04} is defined by equation (28). Values listed for 21.5°C are averages over 4 sets of data (Mills & Ackers, 1979).

(iv) *Analysis of the Roughton-Lyster data*

In the analysis of the data of Roughton & Lyster (1965), the properties of dimers were eliminated due to the high hemoglobin concentration (2 to 3%) so that both MWC models considered here are equivalent. The Roughton & Lyster data were found to provide a good fit to the MWC model assuming equivalent chain affinities, and the MWC parameters obtained were not unexpectedly different from those of the more recent data sets, considering the differences in solution conditions. For the data at pH 7, 19°C, 0.6 M-phosphate the variance of fit was 2.21×10^{-5} as compared with 2.17×10^{-5} for a model-independent fit to the tetramer Adair equation. Allosteric parameters were: ${}^0L_4 = 2.35 \times 10^4$, $c = 1.08 \times 10^{-2}$, $K_R = 7.55 \times 10^5 \text{ mol}^{-1}$. The data at pH 9.1 yielded variances of 3.64×10^{-5} (MWC) versus 4.31×10^{-5} (Adair) with ${}^0L_4 = 2.4 \times 10^2$, $c = 1.7 \times 10^{-2}$, and $K_R = 2.38 \times 10^6 \text{ mol}^{-1}$.

(b) *Hemoglobin Kansas*

The hemoglobin Kansas data (Fig. 2(a)) were first analyzed according to the simple two-state model in which the α and β chains are equivalent and dimers are equivalent to halves of R-state tetramers. The variance of fit obtained with this model (2.3×10^{-4}) was considerably higher than that of the model-independent analysis (1.3×10^{-4}) shown in Figure 2(b) and the distribution of residuals was found to be significantly more non-random. We thus conclude that the two-state model with equivalent α and β chains is inconsistent with the data for the linked dimer-tetramer system of hemoglobin Kansas.

The two-state model discussed above was generalized by two separate modifications and the resulting models tested: (1) functional non-equivalence was allowed in the chains, while retaining the assumption that dimers are equivalent to halves of R-state tetramers. This *modified two-state model* was found to fit the data as well as the thermodynamic linkage functions, as is seen by comparing Figure 2(b) and (d). A variance of 1.3×10^{-4} was obtained with this model. Table 4 shows values of the model parameters obtained. In the second generation of the two-state model we allowed the dimers to exist at a different energy state from R-state tetramers, and also allowed them to assume non-equivalent chain affinities. The

TABLE 4

*Estimates of the allosteric parameters for hemoglobin Kansas
(modified two-state model)*

	ΔG_L	ΔG_R^α	ΔG_T^α	ΔG_R^β	ΔG_T^β	ΔG_c^α	ΔG_c^β
A. <i>Tetramers</i>							
	-8.39	-8.48	-6.08	-7.29	-4.73	2.40	2.57
B. <i>Dimers</i>							
	—	-8.48	-8.48	-7.29	-7.29	0	0

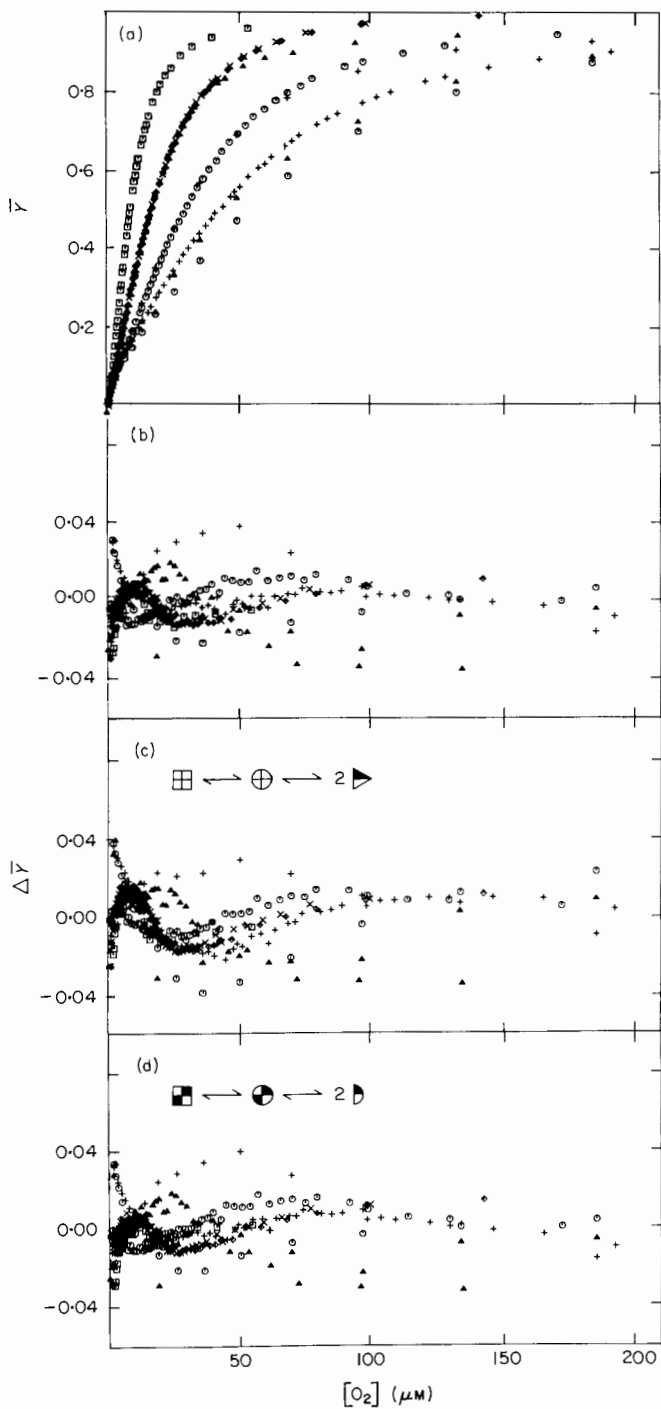


FIG. 2.

chain affinities within tetrameric species, however, were not allowed to differ between α and β . This *modified three-state model* retains the requirement that the tetramers are a simple two-state MWC system but the dimers are no longer halves of R-state tetramers. Results of fitting this model provided a variance of 1.96×10^{-4} , which is significantly larger than that of the thermodynamic fit. The residual plot for this model is shown in Figure 2(c). It may be noted in connection with these results that the model-independent thermodynamic analysis for hemoglobin Kansas does not require a quaternary enhancement effect of the kind seen in hemoglobin A (Atha *et al.*, 1979).

Finally, the Kansas data were fitted to a version of the general model with functional non-equivalence of chains and in which the dimers were allowed to be an allosteric system. The fits to this model resulted in a value of ${}^0L_2 = 0$, so that the modified two-state model in effect resulted.

A striking result of the hemoglobin Kansas analysis (Table 4) is that the allosteric constant for tetramers ($\Delta G_L = -8.4$ kcal) is approximately the same as for hemoglobin A ($\Delta G_L = -9.0$ kcal; Table 2, 21.5°C). This result is at variance with previous assessments (Shulman *et al.*, 1975; Edelstein, 1971) and has an important bearing upon mechanistic interpretations. The low affinity of hemoglobin Kansas tetramer does not arise from an unusually high allosteric constant favoring the T-state species. It is largely the consequence of a greatly reduced oxygen affinity of β chains in the T state, and reduced values of the ratio between affinities in the R and T states. In qualitative agreement with previous analyses is the finding that the transition from predominantly T-state molecules to predominantly R-state molecules occurs at a higher degree of ligation in hemoglobin Kansas. This is shown in Figure 3. The position of the allosteric equilibrium for doubly liganded tetramers in hemoglobin A is approximately the same as for triply liganded tetramers with hemoglobin Kansas. For the Kansas tetramers at this state of ligation, the ratio between T and R forms is only 5.5. By contrast the corresponding ratio for triliganded hemoglobin A is 0.025. The slopes of the two lines in Figure 3 reflect different "rates" of T \rightarrow R transition for the two hemoglobins during ligation. The difference in position of the allosteric equilibrium for the two hemoglobins at a given degree of ligation arises primarily from the difference in the parameter c . The allosteric constant 0L_4 determines the upper endpoint corresponding to no ligation for each plot. It is shown in Figure 3 that these endpoints are very close, whereas the other endpoints, corresponding to complete ligation, differ considerably between the two hemoglobins.

FIG. 2. Analysis of the concentration-dependent oxygenation data of Atha *et al.* (1979) for hemoglobin Kansas. (a) The raw experimental data collected at concentrations between 4×10^{-7} M-heme and 6×10^{-3} M-heme (Atha *et al.*, 1979). (b) The distribution of residuals for the best fit in the previously published thermodynamic analysis (Atha *et al.*, 1979). (c) Distribution of residuals for the fit to a modified 3-state MWC mechanism, where tetramer R and T states have equivalent α and β chains and the dimer has non-equivalent α and β chains. (d) Distribution of residuals for the fit to a modified 2-state MWC mechanism in which α and β chains are not equivalent.

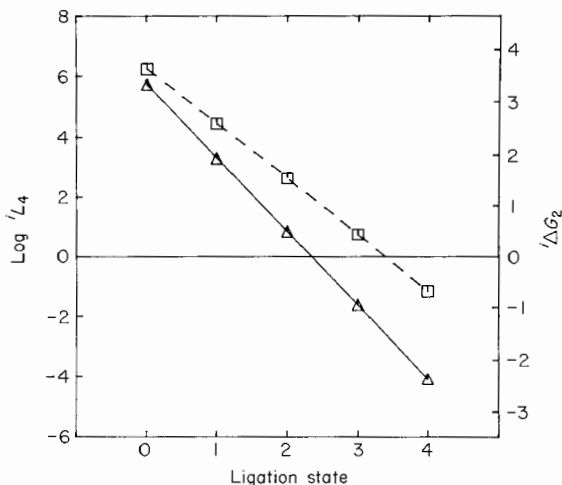


FIG. 3. Allosteric constant iL_4 and free energy of allosteric transition $\Delta G = -RT \ln {}^iL_4$. These quantities are shown as a function of ligation state i for tetrameric hemoglobins A₀ (-△-△-) and Kansas (-□-□-).

6. Discussion

The present study is to our knowledge the most detailed and exacting test of MWC models against experimental data for any regulatory protein. The results provide a number of new insights into the nature of allosteric models of the concerted (MWC) type, and their relationship to real systems. Explicit consideration of the ligand-linked subunit association constants is of particular significance in relation to systems such as human hemoglobin, in which the intersubunit contacts involved are known to undergo major structural change. The extended MWC model for the dimer-tetramer case, which has been developed rigorously here, may serve as a prototype for ligand-linked polymerization models of higher stoichiometry.

Results of this study suggest the need for caution in the use of general models for allosteric regulation in polymerizing systems. The oxygenation-linked dimer-tetramer equilibrium of human hemoglobin is undoubtedly one of the simplest prototypes for the "polysteric linkage" model of Colosimo *et al.* (1976). However, the requirement for a third state in hemoglobin A does not permit the model to fit even this system of lowest-order polymerization. This reinforces the need to treat each system as explicitly as possible.

A second caution, which is underscored by the results of this study, is that of avoiding assumptions regarding the thermodynamic properties of subunits for variant hemoglobins. The allosteric properties for hemoglobin Kansas, which have been accurately determined for the first time in this study, differ markedly from the previous interpretations (Edelstein, 1971; Baldwin, 1976) and the differences have significant implications for allosteric theories of hemoglobin in general. A value of

0L_4 , which is approximately two orders of magnitude larger than that found here (1.9×10^6), was estimated by Edelstein (1971) based on the assumption that isolated α and β chains of normal hemoglobin were equivalent in affinity to the R state of hemoglobin Kansas. The low affinity of hemoglobin Kansas has thus been attributed to a large allosteric constant, favoring the T-state tetramers. Arguments were advanced that the combination of low co-operativity (n) and high allosteric constant of hemoglobin Kansas (placing it on bell-shaped curve of n versus 0L_4 predicted from MWC considerations) is evidence for a general MWC mechanism (Edelstein, 1971). By contrast the corresponding variation of n with 0L_4 for a sequential (induced-fit) model was shown to give a continuously increasing curve. With the corrected value for hemoglobin Kansas, as determined in this study, results no longer favor a concerted mechanism as compared with the sequential one. The role of hemoglobin Kansas in the bell-shaped curve has also been emphasized by Baldwin (1976), who cites the effect of inositol hexaphosphate in reducing the apparent Hill coefficient from 1.6 to 0.9 as evidence for the MWC model. It has been shown, however, that the higher Hill constant under the conditions cited ($60 \mu\text{M}$ -heme) is due almost entirely to the linked dimer-tetramer equilibrium (Atha *et al.*, 1979) and the value of 0.9 results when the equilibrium is shifted to favor tetramers by the presence of inositol hexaphosphate. All of these results underscore the importance of actually determining the thermodynamic properties of subunits and of tetramers for a particular hemoglobin system.

A serious problem lies in establishing correlations between energetic states with "R" and "T" states defined on the basis of crystallographic structures. Since these structures are accurate to only about 2.5 Å, they allow a large range of energy states (e.g. tens of kilocalories) due to the steep variation of potential functions with interatomic distance (see Gelin & Karplus, 1977). Thus a variant hemoglobin (e.g. hemoglobin M Iwate), which is judged by crystallographic criteria to be "frozen in the T state" throughout oxygenation (Greer, 1971), may in fact never be near the same energetic state as that of deoxy hemoglobin A, which is also judged to be "in the T state". This problem of definition of states may account for large differences found in the determined enthalpies of T \rightarrow R transition. A value of 9 kcal was estimated by Gaud *et al.* (1975) for the enthalpy of T \rightarrow R transition in hemoglobin A from a comparison between the heat of ligation for the variant hemoglobin M Iwate (presumably always in the T-state) and the corresponding heat of ligation for hemoglobin A, which included the heat of T \rightarrow R transition. By contrast, a value of 30.6 kcal was obtained in this study from the data obtained on hemoglobin A alone. A likely explanation of this discrepancy is that the crystallographically defined T-state for hemoglobin M Iwate does not represent the same energetic state as that of deoxy hemoglobin A. It should be noted that the states of the MWC model are theoretically defined in terms of thermodynamic quantities rather than structural parameters.

It is worth noting once again that the physical relevance of the MWC models rests mainly upon the experimental finding of at least two major quaternary structures for the tetrameric molecule (Perutz, 1976). The model does not take into account the known changes in tertiary structure that accompany oxygenation of the individual chains, nor the non-covalent bonding interactions between the

subunits that are also known to change upon ligation (see Baldwin & Chothia (1979) for a recent summary of the ligation-linked structure changes). As a first-order approximation, the model has the virtue of requiring a minimum of assumptions and model parameters. On the other hand, the MWC model as applied to hemoglobin generally predicts insensitivity to the most desirable kinds of tests, i.e. physical measurements of the proportion between R and T-state molecules. Severe limitations are placed on any conceivable techniques for such measurement by the vanishingly small fraction of R-state molecules in unliganded hemoglobin (at least under physiological conditions), the vanishingly small fraction of T-state molecules in oxy hemoglobin, and the small fractions of the population having intermediate degrees of ligation and R/T ratios. As experimental information accumulates requiring additional states for tetrameric molecules on model-independent grounds (e.g. see Viggiano & Ho, 1981), it will become necessary to modify, extend, or completely replace such models.

APPENDIX

Definitions and Thermodynamic Relations

The model-independent saturation function for the linked dimer-tetramer system:

$$\bar{Y}_{2,4} = \frac{Z'_2 + Z'_4 (\sqrt{Z_2^2 + 4^0 K_2 Z_4 [P_t]} - Z_2) / 4Z_4}{Z_2 + \sqrt{Z_2^2 + 4^0 K_2 Z_4 [P_t]}}, \quad (\text{A1})$$

where

$$Z_2 = 1 + K_{21}[X] + K_{22}[X]^2 \quad (\text{A2})$$

$$Z'_2 = K_{21}[X] + 2K_{22}[X]^2 \quad (\text{A3})$$

$$Z_4 = 1 + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4 \quad (\text{A4})$$

$$Z'_4 = K_{41}[X] + 2K_{42}[X]^2 + 3K_{43}[X]^3 + 4K_{44}[X]^4, \quad (\text{A5})$$

where

$$K_{2i} = \frac{[(\alpha\beta)X_i]}{[\alpha\beta][X]^i} \quad i = 1, 2 \quad (\text{A6})$$

and

$$K_{4i} = \frac{[(\alpha_2\beta_2)X_i]}{[\alpha_2\beta_2][X]^i} \quad i = 1, 2, 3, 4. \quad (\text{A7})$$

The sequential binding constants are defined as:

$$k_{21} = \frac{[(\alpha\beta)X_i]}{[(\alpha\beta)X_{i-1}][X]} = \frac{K_{2i}}{K_{2(i-1)}} \quad (K_{20} = 1) \quad (\text{A8})$$

$$k_{4i} = \frac{[(\alpha_2\beta_2)X_i]}{[(\alpha_2\beta_2)X_{i-1}][X]} = \frac{K_{4i}}{K_{4(i-1)}} \quad (K_{40} = 1). \quad (\text{A9})$$

The sequential binding free energy changes are defined in terms of the sequential binding constants as:

$$\Delta G_{n,i} = RT \ln k_{n,i}. \quad (\text{A10})$$

The subunit association constants iK_2 are defined as the dimer-tetramer association constants, per mole of dimer, to form a tetramer with i oxygens bound:

$${}^iK_2 = \frac{[(\alpha_2\beta_2)X_i]}{[(\alpha\beta)X_j][(\alpha\beta)X_k]}; \quad \begin{matrix} i = j+k \\ j, k = 0, 1, 2 \end{matrix} \quad (\text{A11})$$

The corresponding subunit association free energy changes are defined as:

$${}^i\Delta G_2 = -RT \ln {}^iK_2. \quad (\text{A12})$$

For a more complete definition of terms, see the previously published discussions (Ackers & Halvorson, 1974; Mills *et al.*, 1976; Johnson *et al.*, 1976).

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