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ARTICLE *in* BIOCHEMISTRY · JANUARY 1982

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## Thermodynamic Analysis of Human Hemoglobins in Terms of the Perutz Mechanism: Extensions of the Szabo-Karplus Model To Include Subunit Assembly<sup>†</sup>

Michael L. Johnson\* and Gary K. Ackers

**ABSTRACT:** The stereochemical postulates of Perutz for the mechanism of hemoglobin [Perutz, M. F. (1970) *Nature (London)* 228, 726-739] have been formulated into a statistical thermodynamic model. The model is based on that of Szabo and Karplus [Szabo, A., & Karplus, M. (1972) *J. Mol. Biol.* 72, 163-197] but has been extended to include the properties of dissociated dimers in equilibrium with tetramers. The dissociation eliminates the  $\alpha_1\beta_2$  intersubunit contact which is the major site of ligand-linked structure change. The model quantitatively describes the coupling between binding of oxygen and protons in dimers and tetramers, the change in quaternary structure, and the breaking of salt bridges which are assumed to stabilize the deoxy quaternary structure. The extended model has been tested against an extensive series of recent experimental data from our laboratory and elsewhere

on the ligand-linked dimer-tetramer assembly in normal human hemoglobin A and in the variant hemoglobin Kansas ( $\beta 102 \text{ Asp} \rightarrow \text{Asn}$ ). Two versions of the model were used which differ in the properties of the dissociated dimers. For both hemoglobins, the models were found capable of simultaneously describing the data on the ligand-linked dimer-tetramer assembly and predicting the tetramer Bohr effect. However, neither model predicted reasonable values for the tetramer Bohr effect without simultaneously predicting unreasonable values for the affinities of individual chains. Both models incorrectly predict preferential binding of oxygen to the  $\alpha$  or  $\beta$  chains within the tetramer. These results argue against the Perutz mechanism for the molecular processes of hemoglobin.

The elegant structure work of Perutz et al. on the deoxy and ligated forms of hemoglobin (Perutz et al., 1968, 1969; Bolten & Perutz, 1970) led him to propose a mechanism of cooperativity in which various parts of the molecule act as a system of levers (Perutz, 1970a,b; cf. Perutz, 1976, 1979, for recent reviews). This mechanism prescribes a set of specific rules for the transmission of stereochemical effects between the oxygen-binding heme sites and a set of oxygenation-sensitive salt bridges which link subunits of the tetrameric structure. The rules also prescribe a rationale for the Bohr effect based on a small number of ionizable groups.

The Perutz mechanism has been found consistent with many of the observed properties of normal and abnormal hemoglobins and is presented in several textbooks as the definitive solution to the problem of hemoglobin function (cf. Stryer, 1981; Cantor & Schimmel, 1980). Critical testing of this theory, however, has awaited the development of more accurate and sensitive techniques and of more extensive experimental data reflecting physical properties of hemoglobin at the in-

termediate states of oxygenation. Recent studies using the techniques of EXAFS (Eisenberger et al., 1976, 1978), resonance Raman (Nagai et al., 1978), and NMR spectroscopy (Viggiano & Ho, 1979; Russu et al., 1980) have provided critical tests of certain aspects of the Perutz mechanism. Thermodynamic information provides a basis for testing certain other aspects. In the present study, we have analyzed recent thermodynamic results for normal human hemoglobin and for the variant hemoglobin Kansas ( $\beta 102 \text{ Asp} \rightarrow \text{Asn}$ ) in terms of quantitative models which embody the proposals of Perutz regarding the roles of salt bridges and ionizable Bohr groups.

The Perutz mechanism is based upon the following assumptions: (a) The tetramer exists in either of two quaternary structures with no intermediate forms. (b) The unliganded tetramer has greater stability than the oxygenated molecule due to the energies of additional bonds which consist of salt bridges across the  $\alpha_1\beta_2$  interface. (c) The heme iron moves into the plane of the porphyrin ring upon oxygenation, acting as a trigger for the tertiary structure change in the respective  $\alpha$  or  $\beta$  chains. (d) The tertiary structure changes of the chains lead to the breaking of the intersubunit salt bridges and also of two intrasubunit salt bridges within the  $\beta$  chains. (e) The Bohr effect originates in the ionization of two sets of groups associated with salt bridges which are broken upon oxygenation. Within the context of these assumptions, the Perutz mechanism defines a set of specific rules (to be discussed) for

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the structural and chemical transformations which accompany the functional cycle of the hemoglobin molecule. Tests of the mechanism may thus be considered at two levels: (a) that of the general assumptions listed above and (b) that of the specific rules which are prescribed to operate within the framework of the general assumptions. The work described in this paper has a direct bearing only on the specific formulation of the Perutz mechanism (level b). The relationship of this and other recent work to the general assumptions (level a) are considered under Discussion.

The Perutz mechanism is based largely upon structural proposals whereas the driving forces for the functional transitions of the hemoglobin molecule must be energetic in character. An important role of models is to correlate the structural, energetic, and functional properties of macromolecular systems. Among attempts to bridge the gap between structural and functional information on human hemoglobin, an important advance was made by the work of Szabo & Karplus (1972). These authors formulated a statistical thermodynamic model for correlating structural changes which accompany the binding of oxygen and protons by tetrameric human hemoglobin with the observable solution properties. Their model provided a translation of the structural proposals of Perutz (1970a,b) into the observable binding properties for oxygen and protons. The Perutz proposals were based largely upon the X-ray analysis of crystal structures corresponding to the unligated and fully ligated states of the tetrameric molecule. A major value of statistical thermodynamic models is their ability to predict properties of the molecules at intermediate states of ligation [cf. Weber (1972) and Herzfeld & Stanley (1974) for other statistical thermodynamic models of hemoglobin].

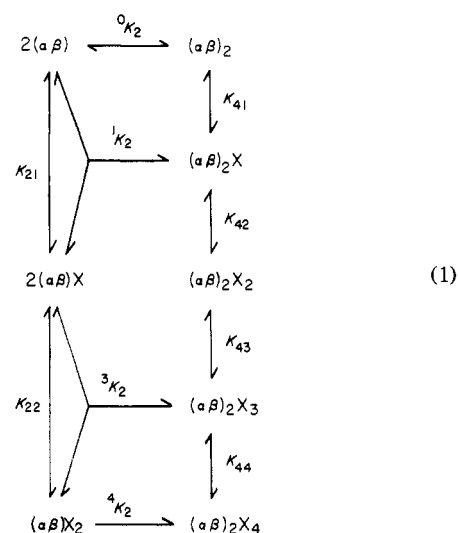
Szabo and Karplus carried out least-squares fits of their model to the experimental oxygen binding data of Roughton & Lyster (1965), at pH 7.0 and 9.1. They were able to account for the available experimental results by using values of the model parameters (e.g., free energies of salt bridge formation, quaternary structure change, etc.) which were within physically reasonable ranges. However, critical tests of the Perutz/Szabo-Karplus concepts have required the development of a more extensive and comprehensive base of experimental thermodynamic information than was afforded by the Roughton-Lyster data. In particular, the need to probe the system in other ways than by tetramer oxygenation curves was evident.

Of special importance is experimental data, such as that analyzed in the present study, which directly reflect the energies of ligand-linked quaternary structure changes in the hemoglobin tetramer. In the Perutz mechanism, many of the important structural features which change upon oxygenation are localized in the  $\alpha_1\beta_2$  intersubunit contact, particularly the salt bridges which are believed to play a dominant role in stabilizing the deoxy quaternary structure. On model-independent grounds, the  $\alpha_1\beta_2$  contact was found to be the site of the major structure changes accompanying oxygenation (cf. Perutz, 1976). These considerations motivated us to investigate the stability of the  $\alpha_1\beta_2$  contact as a function of oxygenation state (cf. Ackers & Halvorson (1974) for a discussion of rationale). During the last 7 years, an extensive base of experimental information has been obtained for the linkage between oxygenation and subunit dissociation. The data pertain to normal human hemoglobin (Ip et al., 1976; Mills et al., 1976, 1979; Johnson et al., 1976; Valdes et al., 1978; Ip & Ackers, 1977; Mills & Ackers, 1979; Chu & Ackers, 1981; A. Chu, B. W. Turner, and G. K. Ackers, unpublished

results) and to the variant Hemoglobin Kansas (Atha & Riggs, 1976; Atha et al., 1979). Applying the concepts previously developed for tetramers by Szabo and Karplus, we have carried out extensions of their model to take into account the properties of dimers in equilibrium with the tetrameric hemoglobin molecules. This extension was required to analyze the experimental oxygen binding curves which reflect the dissociation of tetramers into dimers in various states of oxygenation. This new dimension of analysis is of particular importance since it directly reflects the energies of ligand-linked structure change within tetrameric hemoglobin. The extended models have been subjected to rigorous testing by numerical analysis against the experimental data on oxygen binding and dimer-tetramer assembly. In assessing the physical relevance of the fitted model parameters, we have also taken into account (a) the magnitude of the Bohr effect for tetramers independently determined under the same experimental conditions and (b) recent information on the relative affinities for oxygen of the  $\alpha$  and  $\beta$  chains within the tetrameric molecule determined under comparable conditions by nuclear magnetic resonance studies (Johnson & Ho, 1974; Viggiano & Ho, 1979).

### Theory

In this section, we show how the statistical thermodynamic approach used by Szabo and Karplus can be extended to the case of ligand-linked dissociation and how the statistical thermodynamic parameters are translated into the basic thermodynamic constants of the dimer-tetramer linkage, as depicted in eq 1. In mechanism 1,  $^iK_2$  are the equilibrium



constants for the formation of tetramers with  $i$  oxygens,  $X_i$ , bound. The tetramers are formed from the appropriate combinations of dimers.  $K_{2i}$  and  $K_{4i}$  are the product (Adair) binding constants for each stage of ligation. Previous papers contain more detailed discussion and elaboration of the properties of the linkage scheme (Ackers & Halvorson, 1974; Mills et al., 1976; Johnson et al., 1976; Johnson & Ackers, 1977; Valdes et al., 1978; Mills & Ackers, 1979). Here we present only a brief statement of the relationships to be used in the model analyses.

(A) *Model-Independent Binding Isotherm.* The mathematical form of the binding isotherm for the ligand-linked dimer-tetramer association system of mechanism 1 is (Ackers & Halvorson, 1974)

$$\bar{Y}_{2,4} = \frac{Z_2' + Z_4'(\sqrt{Z_2^2 + 4^0K_2Z_4[P]} - Z_2)/(4Z_4)}{Z_2 + \sqrt{Z_2^2 + 4^0K_2Z_4[P]}} \quad (2)$$

where

$$Z_2 = 1 + K_{21}[X] + K_{22}[X]^2 \quad (3)$$

$$Z_2' = K_{21}[X] + 2K_{22}[X]^2 \quad (4)$$

$$Z_4 = 1 + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4 \quad (5)$$

$$Z_4' = K_{41}[X] + 2K_{42}[X]^2 + 3K_{43}[X]^3 + 4K_{44}[X]^4 \quad (6)$$

$[P_i]$  is the total protein concentration in molar heme,  $^0K_2$  is the subunit association constant to form unliganded tetramers from unliganded dimers,  $[X]$  is the molar oxygen concentration, and  $K_{21}$  and  $K_{41}$  are the product Adair constants for dimers and tetramers, respectively. Formulation of any model for the hemoglobin system depicted in mechanism 1 consists of defining the relationships between the parameters of the particular model and the model-independent phenomenological eq 2-6 (Ackers & Johnson, 1981). In section C, we show how these relationships are established for the Szabo-Karplus model. In section B, we briefly summarize the assumptions of their model. For a more detailed description one should consult the original paper (Szabo & Karplus, 1972).

(B) *Szabo-Karplus Model.* We use essentially the same terminology as Szabo and Karplus but will define several additional terms to represent energies or equilibrium constants where convenient. The specific rules regarding properties of the tetrameric molecule as formulated by Szabo & Karplus (1972) are summarized as follows:

(1) The hemoglobin tetramer has two quaternary structures, (the oxy and deoxy forms) defined such that the oxy form is more stable than the deoxy form whenever the equilibrium constant  $Q$  (for quaternary structure equilibrium) is smaller than unity. The free-energy difference between the deoxy and oxy quaternary forms is  $\delta_Q = -RT \ln Q$ . ( $\delta$ 's are used throughout this paper to denote free energies of various processes).

(2) Each of the  $\alpha$  and  $\beta$  chains has two tertiary structures, the liganded and the unliganded forms, with the individual free-energy differences  $\delta_{\alpha\alpha} = -RT \ln K_\alpha$  and  $\delta_{\beta\beta} = -RT \ln K_\beta$ , defined to include the  $O_2$  binding.

(3) (i) In the deoxygenated quaternary structure, a number of interchain and intrachain salt bridges can exist. Each  $\alpha$  chain, in its unliganded form, can have two salt bridges, with free energies of  $\delta_{\alpha\alpha} = -RT \ln S_{\alpha\alpha}$ , and  $\delta_{\alpha\alpha}^H = -RT \ln S_{\alpha\alpha}^H$ , to the other  $\alpha$  chain. Each  $\beta$  chain, in its unliganded form, can have one salt bridge, with the energy of  $\delta_{\alpha\beta} = -RT \ln S_{\alpha\beta}$  to one of the  $\alpha$  chains ( $\beta_1$  to  $\alpha_2$  and  $\beta_2$  to  $\alpha_1$ ), and one internal salt bridge, with free energy of  $\delta_\beta^H = -RT \ln S_\beta^H$ . In the liganded form of chains, the salt bridges defined as originating in the liganded chain are broken. In the oxygenated quaternary structure, intrachain salt bridges can exist, but interchain salt bridges cannot. (ii) Two of the four salt bridges connecting the  $\alpha$  chains involve  $NH_3^+$  groups with ionizable protons which also contribute to the Bohr effect. The two intrachain salt bridges of the  $\beta$  chains involve ionizable imidazole  $NH^+$  groups which also contribute. In the Szabo-Karplus formulation, it is assumed that (a) the protons can be ionized only when their salt bridges are broken and (b) the protons released are absorbed by hydroxyl ions. The net free energies for release of the Bohr protons and their absorption by  $OH^-$  are given by  $\delta_\alpha^H = -RT \ln H_\alpha$  and  $\delta_\beta^H = -RT \ln H_\beta$  for the two kinds of Bohr groups. For each ionizing group, it is assumed that the corresponding salt bridge does not form in the absence of the ionizable proton.

(4) Certain very low probability structures are ignored. Examples are the liganded tertiary structure for individual

chains in the absence of bound ligand and broken salt bridges where they are permitted.

Although this model is not a "perfect translation" of the Perutz proposals, as pointed out by Szabo & Karplus (1972), it does provide a close approximation and embodies the essential features of the Perutz mechanism, particularly with regard to the quaternary structure change, the role of salt bridges, and the Bohr protons. It does not permit explicit tests of heme iron movement upon ligation, since all events within a subunit chain are contained in the parameters  $K_\alpha$  and  $K_\beta$ .

Within the framework of the assumptions stated above, Szabo and Karplus formulated a generating function  $\Xi_4$  which is the macroscopic analogue of the grand canonical partition function. They treated the hemoglobin system as an absorber of both oxygen and hydroxyl ions. We have treated the hemoglobin molecule instead as an absorber of hydrogen ions (i.e., the Bohr protons themselves) and oxygen. The formulations are algebraically equivalent when the corresponding definitions are used for the energetic contributions due to the bound and unbound species. When the protons and oxygen are used as ligands, the partition function of hemoglobin tetramers is

$$\Xi_4 = \sum_i g_{ij} e^{-G_{ij}/(RT)} [H^+]^{m_{ij}} [X]^i \quad (7)$$

where  $G_{ij}$  are free energies of the various microscopic configurations of the molecule with  $i$  oxygens bound,  $j$  is an index for each distinguishable microscopic configuration of the molecule with  $i$  oxygens bound, and  $g_{ij}$  is the statistical degeneracy. In eq 7,  $[H^+]$  and  $[X]$  are concentrations of the unbound ligands and  $m_{ij}$  is the stoichiometry of proton binding corresponding to each species. For the Boltzmann factors of the partition function, Szabo and Karplus used the equilibrium constants defined for the various processes of the model and were able to reduce the partition function to a compact analytic form. Thermodynamic properties of the model are then deduced from the appropriate derivatives of the resulting generating function.

It is of interest to note that this model requires a total of 512 configurations of the hemoglobin tetramer, which are enumerated as follows: There are 32 ways of forming an unliganded tetramer, 18 of which are nonredundant (i.e., distinguishable from each other). Singly liganded tetramer can be formed in 128 ways, 45 of which are not redundant. The largest population is the doubly liganded tetramer which can be formed in 192 ways, 64 of which are different. The triply liganded species have the same number of configurations as the singly liganded ones, and the fully liganded set is equal in number to the unliganded set. Thus the total number of configurations is 512, of which 190 are different. We have written a computer program which enumerates all the microscopic configurations and their degeneracies. A listing of these species is given in Appendix A of the supplementary material (see paragraph at end of paper regarding supplementary material).

The above enumeration of rules and species is illustrative of the components which are necessary to any statistical thermodynamic model: (a) a specification of the pairwise energetic constraints within and between the parts of the system and (b) a set of rules for alteration of these constraints with ligand binding. In general both (a) and (b) are powerful in limiting the number of possibilities for operation of the system. One might suppose, for example, that a model with 190 species could be made to fit virtually any experimental data. This is not so when the rules are very stringent and very specific, as in the case of this model, and when the model

parameters are required to have values which lie within physically reasonable ranges. In addition, the Szabo-Karplus formulation involves simplifying assumptions, such as the salt bridges having equal energies. These simplifying assumptions and the existence of symmetry properties have the effect of reducing the number of possible states. The final step of their formulation takes advantage of the symmetry of the various constraints to group the partition function into small summations and to eliminate the large number of terms.

(C) *Formulation of the Extended Model.* In extending the model of Szabo and Karplus, we have used a different computational approach. Since we wished to treat a larger system of species and to eliminate the restriction of identical salt bridge energies, we have not used the analytical form of the partition function and have used a different method for grouping the terms of this function. Our formulation for the tetrameric states becomes algebraically equivalent to the Szabo-Karplus model when the restrictions are imposed. The computational method we used also provides a determination of the confidence limits on values of the estimated parameters (Johnson et al., 1976).

In order to estimate the values of  $K_{ni}$  for use with eq 2-6, we evaluated the free energy,  $G_{ij}$ , for each molecular species relative to a reference state. For tetramers, we used the same reference state as Szabo and Karplus, i.e., the oxy quaternary structure with the  $\alpha$  and  $\beta$  chains in the unliganded tertiary structure. The dimer reference state is discussed later. We define the quantity  $\xi_{ni}$  which is a macroscopic analogue of the grand canonical partition function (cf. Hill, 1960) for the subsystem comprised of  $n$ -mers with  $i$  ligands bound:

$$\xi_{ni} = \sum_j g_{ij} e^{-G_{ij}/(RT)} [H^+]^{m_{ij}} \quad (8)$$

The relationship between the subsystem partition function  $\xi_{ni}$  and the other system properties is readily seen by noting that the expression on the right of eq 7 may be written as a sum of terms in increasing powers of  $[X]$ , from 0 to 4.

$$\Xi_4 = \xi_{40} + \xi_{41}[X] + \xi_{42}[X]^2 + \xi_{43}[X]^3 + \xi_{44}[X]^4 \quad (9)$$

A term by term comparison of this expression with eq 5, which is a macroscopic analogue of the grand partition function in terms of product Adair constants, yields

$$K_{4i} = \frac{\xi_{4i}}{\xi_{40}} \quad (10)$$

Similar relations may be written for dimers. The resulting values of  $K_{ni}$  are related to the average free energies  $G_{ni}$  according to

$$K_{ni} = \exp[(G_{n0} - G_{ni})/(RT)] \quad (11)$$

$$G_{ni} = RT \ln \xi_{ni} \quad (12)$$

The grand canonical partition function for the complete linked dimer-tetramer system is

$$\Xi = [D]\Xi_2 + 2^0 K_2 [D]^2 \Xi_4 \quad (13)$$

where  $\Xi_2$  is the partition function for dimers in all ligation states and  $[D]$  is the concentration of dissociated dimers. Within the framework of these relationships, the remaining requirement is to evaluate the free energy  $G_{ij}$  of each microscopic configuration of tetramers or dimers. These energies can each be expressed as a sum of four possible terms:

$$G_{ij} = G_s + G_H + G_X + G_Q \quad (14)$$

where the subscripts (s, H, X, and Q) refer to the type of energetic constraint on the molecule relative to the reference state. The constraints due to salt bridges,  $G_s$ , are the sum of

energetic terms for each of the salt bridges:

$$G_s = n_{\alpha\alpha}\delta_{\alpha\alpha} + n_{\alpha\beta}\delta_{\alpha\beta} + n_{\beta\beta}\delta_{\beta\beta} \quad (15)$$

where  $\delta_{\alpha\alpha}$ ,  $\delta_{\alpha\beta}$ , and  $\delta_{\beta\beta}$  refer to the free energies of formation for the three types of salt bridge and  $n_{\alpha\alpha}$ ,  $n_{\alpha\beta}$ , and  $n_{\beta\beta}$  denote the number of each of the salt bridges in the particular configuration  $ij$ . The free energy due to proton binding,  $G_H$ , is given as

$$G_H = n_{\alpha}^H \delta_{\alpha}^H + n_{\beta}^H \delta_{\beta}^H \quad (16)$$

where  $\delta_{\alpha}^H$  and  $\delta_{\beta}^H$  are the energies of binding a proton to the Bohr groups on an  $\alpha$  or  $\beta$  chain;  $n_{\alpha}^H$  and  $n_{\beta}^H$  are the number of protons bound. The free energy increment due to the bound oxygens,  $G_X$ , is

$$G_X = n_{4\alpha}\delta_{4\alpha} + n_{4\beta}\delta_{4\beta} \quad (17)$$

where  $\delta_{4\alpha}$  and  $\delta_{4\beta}$  represent, for the two types of chains, the intrinsic free energies of oxygen binding plus the energies of associated tertiary structure change;  $n_{4\alpha}$  and  $n_{4\beta}$  are the numbers of oxygens on an  $\alpha$  chain or a  $\beta$  chain, respectively. The subscript 4 refers to tetramer. Similar relations are written for the dissociated dimers. It should be noted that the dimers and tetramers may have different intrinsic affinities for oxygen.

For each microscopic configuration of tetramers, the free energy contribution  $G_Q$  due to the quaternary structure is equal to zero if the configuration of the molecule has the oxy quaternary structure or  $\delta_Q$  if the molecule is in the deoxy quaternary structure.

When these procedures are used, then all of the terms of eq 7, 8, or 13 may be readily evaluated. The combination of eq 8, 10, and 2 (with the dimer versions of eq 10) provides the complete translation between the model-independent binding isotherm for the linked dimer-tetramer system and the extended versions of the Szabo-Karplus model.

Evaluation of the terms in the partition function also permits a calculation of the probability for each distinguishable microscopic form of the molecule:

$$P_{ij} = \frac{g_{ij} e^{-G_{ij}/(RT)} [H^+]^{m_{ij}} [X]^i}{\Xi} \quad (18)$$

Within a given oxygenation state for dimers or tetramers, the fraction of molecules that have the microscopic configuration  $ij$  is

$$f_{ij} = \frac{g_{ij} e^{-G_{ij}/(RT)} [H^+]^{m_{ij}}}{\xi_{ni}} \quad (19)$$

Explicit calculation of the number of Bohr protons bound at each stage of oxygenation is carried out according to

$$\bar{\nu}_{ni}^{H^+} = \sum f_{ij} (n_{\alpha}^{H,ij} + n_{\beta}^{H,ij}) \quad (20)$$

and the number of Bohr protons released upon fully oxygenating the molecule (dimer or tetramer) is given by the difference  $\bar{\nu}_{44}^{H^+} - \bar{\nu}_{40}^{H^+}$ . The average energy of Bohr proton binding to the hemoglobin molecules having  $i$  oxygens bound is

$$\delta_{ni} = \sum f_{ij} G_H \quad (21)$$

where  $G_H$  is given by eq 16. The free energy of Bohr proton release which accompanies the complete oxygenation of tetramers is given by

$$\bar{\delta}_{H^+} = \delta_{44} - \delta_{40} \quad (22)$$

The method we have used in formulating the model can also be used to impose complex constraints on relationships between the model parameter values. For example, the fraction of

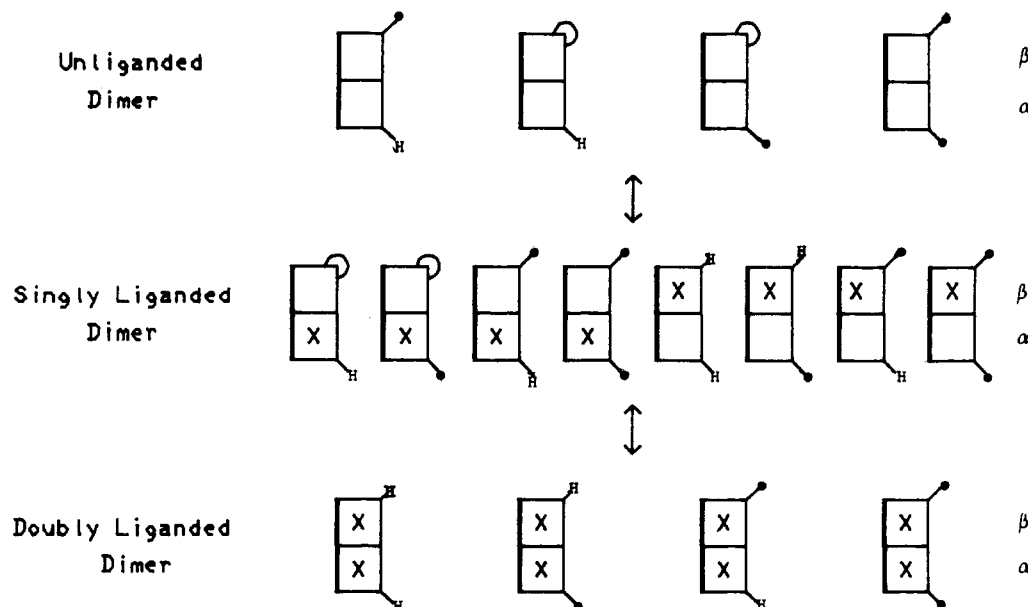


FIGURE 1: Configurations of dimers for the constrained dimer model. Subunits within the dimer are represented by squares ( $\beta$  above and  $\alpha$  below) joined at the  $\alpha_1\beta_1$  interface. Heavy vertical sides of the dimer represent the dissociated  $\alpha_1\beta_2$  interface. Bound oxygen molecules are represented by X within the subunit. The intrachain salt bridge is denoted by an arc on the  $\beta$  chains. When the salt bridge is broken but the Bohr proton associated with it is not released, there is a  $-H$ . When the  $\alpha$  chains Bohr proton has not been released, there is a  $-H$ . When either site has released its Bohr proton these symbols are replaced by  $\bullet$ .

bound oxygens which occupy an  $\alpha$  (or  $\beta$ ) chain at intermediate states of ligation can be constrained to 0.5 (to conform with the NMR results: Viggiano & Ho, 1974; Johnson & Ho, 1974). To do this, we write

$$\frac{\sum (n_{4\alpha} - n_{4\beta}) g_{ij} e^{-G_{ij}/(RT)} [H^+]^{m_{ij}}}{\xi_{4i}} = 0 \quad (23)$$

The left side of eq 23 specifies the average difference in number of oxygens bound to the  $\alpha$  and  $\beta$  chains at a particular stage of ligation,  $i$ . In order to impose eq 23 as a constraint on the analysis of data, we use the simultaneous roots of eq 23 at two stages of ligation  $i = 1$  and  $i = 3$  and stipulate that two of the model parameters, e.g.,  $\delta_{4\alpha}$  and  $\delta_{4\alpha}^H$  be functions of the other model parameters; i.e., the values of  $\delta_{4\alpha}$  and  $\delta_{4\alpha}^H$  are determined such that eq 23 is simultaneously satisfied for  $i = 1$  and  $i = 3$ .

(D) *Properties of Dimeric Species.* Two models for the dimeric species were employed in this study. (i) The "unconstrained dimer" model assumes that the intrachain salt bridges of the  $\beta$  chains are eliminated upon dissociation along with the other salt bridges. In this model, the only energetic contributions to the dimers originate in the binding of oxygen, giving rise to a total of four nonredundant stages. (ii) The "constrained dimer" is shown in Figure 1 in all of its microscopic configurations. In this model, it was assumed that dissociation of the tetramer leads to disruption only of the salt bridges which cross the dimer-dimer interface, but the intrachain salt bridge ( $\beta 145$  His- $\alpha 93$  Asp) is not eliminated by dissociation to dimers. This salt bridge follows the same rules in the dimer as in the tetramer; i.e., it is broken if a proton is released or if the  $\beta$  chain is oxygenated. The different energetic states for dimers arise then from the binding of protons and oxygen molecules. No quaternary structure changes are assumed for dimers. This model gives a total of 16 nonredundant states, 4 each for unliganded and doubly liganded dimer and 8 for singly liganded dimer. Again, the intrinsic binding constants of the  $\alpha$  and  $\beta$  chains are not assumed to be equal to each other nor to those of the tetramer.

The macroscopic product Adair constant  $K_{2i}$  of each dimeric species was evaluated for these models by a procedure anal-

ogous to that used for the tetramer constants, eq 12 and 14-17. The reference state for evaluation of the  $G_{ij}$  terms for dimers was the unliganded molecule in both models. For model ii, the reference state used was the species with no oxygens or protons bound and with the salt bridge formed. A complete listing of the microscopic states of tetramers and dimers along with the statistical degeneracy of each state is given in Appendix A of the supplementary material.

#### Numerical Methods

The computational program used in this study has been described elsewhere (Ackers & Johnson, 1981). The program carries out nonlinear least-squares analyses of experimental data in terms of parameters from any particular model that is specified. For each model, a subroutine is written which translates the model parameters into the seven 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 or 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 error statistics as described extensively elsewhere (Johnson et al., 1976).

In judging the goodness of fit for each of the models to the experimental data, we imposed the same criteria as was used in obtaining the best fits to the basic thermodynamic parameters of the linkage system. The assessments were based upon (a) a comparison of the variance of the fit with the variance found in determining the thermodynamic constants, (b) randomness in the distribution of residuals to the best fit, (c) cross-correlation coefficients between the fitted parameters, and (d) comparison between values of the thermodynamic constants calculated by using the best-fit model parameters and the error limits for their values obtained from fitting the same data for the thermodynamic constants alone. In addition to the above criteria used in evaluating the numerical fitting, we also have applied the following test for validity of the results: (e) values of the model parameters or effects arising from them (e.g., the Bohr effect) were required to lie within physically reasonable ranges. For certain of the parameters where assignment of these ranges cannot be made with ac-

curacy, we allowed a wide tolerance to their acceptable limits. For the salt bridges, we assumed the energies to lie in the range 0 to  $-5$  kcal (cf. Szabo & Karplus, 1972). For the intrinsic binding energies  $\delta_{4\alpha}$ ,  $\delta_{4\beta}$ ,  $\delta_{2\alpha}$ , and  $\delta_{2\beta}$ , we assumed limits of  $-7$  to  $-10$  kcal for normal  $\alpha$  and  $\beta$  chains; the experimental values for isolated chains range between  $-8.6$  and  $-7.7$  kcal under these same conditions (Mills & Ackers, 1979). For Kansas  $\beta$  chains, we considered limits of  $-6$  to  $-9$  kcal, with the experimental value being  $-7.5$  kcal (Atha et al., 1979). We did not impose any specific limits on the values of  $\delta_{\alpha}^H$  and  $\delta_{\beta}^H$  but required the magnitude of the total tetramer Bohr effect to lie within ranges (specified for each data set) which correspond to the independently known Bohr effect values. For values of  $Q$ , we allowed any values less than  $\pm 20$  kcal. It should be noted that these limits were not imposed in the fitting itself—only in our evaluation of the results of the fits. The assessments were also based upon (f) examination of the prediction of the model for agreement with available independent data. These data included the relative distribution of oxygens on the  $\alpha$  and  $\beta$  chains at intermediate states of oxygenation (Viggiano & Ho, 1979) and the tetramer Bohr effect values (A. Chu, B. W. Turner, and G. K. Ackers, unpublished results).

The model of Szabo and Karplus as extended to include dimeric species involves 11 model parameters. The parameters we employed in the analysis were the following:  $^0\Delta G_2$ , the subunit association constant of unliganded dimers to form unliganded tetramers;  $\delta_{2\alpha}$  and  $\delta_{2\beta}$ , the intrinsic binding affinities of the  $\alpha$  and  $\beta$  chains of the dimeric species;  $\delta_{4\alpha}$  and  $\delta_{4\beta}$ , the intrinsic affinities of tetramers;  $\delta_{\alpha\alpha}$ ,  $\delta_{\alpha\beta}$ , and  $\delta_{\beta\beta}$ , the free energies of salt bridge formation;  $\delta_{\alpha}^H$  and  $\delta_{\beta}^H$ , the energies for binding the dissociable proton to  $\alpha$  and  $\beta$  chains;  $\delta_Q$ , the energy of the quaternary transition.

#### Data Analyzed

Experimental data analyzed in this study include the following:

**Hemoglobin A.** (a) The data of Roughton & Lyster (1965). Their experiments were performed at sufficiently high hemoglobin concentration to obviate the effects of dimeric species. Since these data were analyzed by Szabo and Karplus, we have used it to compare our program and model with their results. The Roughton-Lyster data consist of two oxygenation curves obtained respectively at pH 7 in 0.6 M phosphate and at pH 9.1 in 0.2 M borate. The temperature for both was 19 °C. (b) The oxygenation data of Mills et al. (1976) consisting of oxygenation curves measured as a function of hemoglobin concentration and temperature. These data were analyzed in combination with (c) the independent results on dimer-tetramer association of unliganded and fully oxygenated hemoglobin (Ip et al., 1976; Ip & Ackers, 1977) obtained under the same conditions: pH 7.4, 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM Na<sub>2</sub>EDTA, 21.5 °C. The data of Mills et al. (1976) is shown in Figure 2 and is listed in Appendix B of the supplementary material. (d) Results of additional recent studies on the tetramer Bohr effect measured under identical conditions with those of Mills et al. (1976). The value obtained for the number of protons released upon complete oxygenation of tetramers was  $0.51 \pm 0.01$  mol of H<sup>+</sup>/mol of heme (A. Chu, B. W. Turner, and G. K. Ackers, unpublished results). In the model analysis reported here, we allowed a much broader range (0.45–0.6) for the calculated magnitude of the tetramer Bohr effect at pH 7.4.

**Hemoglobin Kansas.** We have analyzed the concentration-dependent oxygen binding data of Atha et al. (1979) combined with the data of Atha & Riggs (1976) on the di-

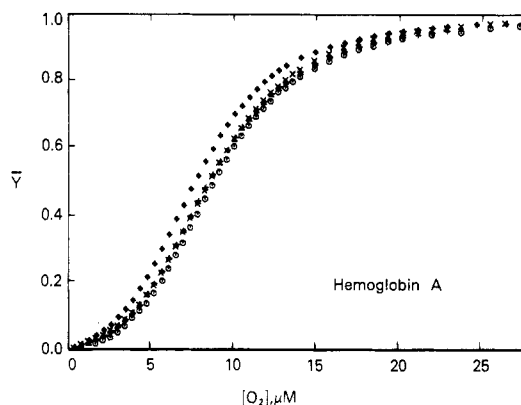


FIGURE 2: Oxygenation binding curves as a function of hemoglobin A concentration, from Mills et al. (1976). These data were measured at heme concentrations between  $4 \times 10^{-4}$  M and  $4 \times 10^{-6}$  M in 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM Na<sub>2</sub>EDTA (pH 7.4), 21.5 °C.

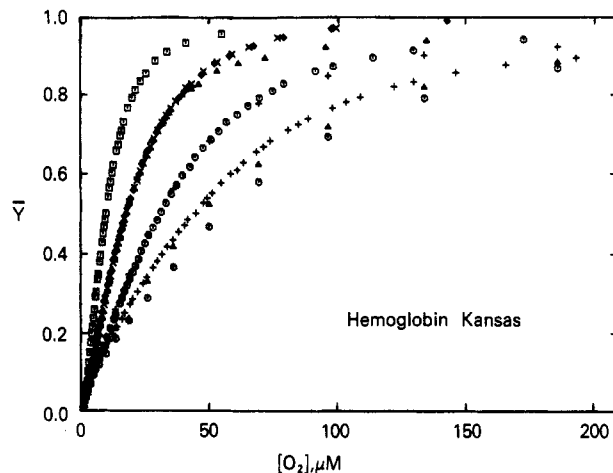


FIGURE 3: Oxygenation binding curves as a function of hemoglobin Kansas concentration, from Atha et al. (1979). These data were measured at heme concentrations between  $4 \times 10^{-4}$  M and  $5 \times 10^{-6}$  M in 0.05 M Tris-HCl, 0.1 M NaCl, and 1 mM Na<sub>2</sub>EDTA (pH 7.5), 20 °C.

mer-tetramer association constants. These data, which are presented in Figure 3, were obtained at pH 7.5 in 0.05 M Tris-HCl, 0.1 M NaCl, and 1 mM Na<sub>2</sub>EDTA at 20 °C. The experimental values for the oxygenation data of Atha et al. (1979) are given in Appendix C of the supplementary material.

#### Results

**(A) Data of Roughton and Lyster.** The hemoglobin A data of Roughton & Lyster (1965) were analyzed by Szabo & Karplus (1972) to evaluate the parameters of their model. In order to test our formulation of the model for tetrameric properties, we carried out analyses by using the Roughton-Lyster data. We have repeated all of the calculations described by Szabo & Karplus (1972) and obtained essentially the same results as they reported within the confidence limits estimated by our program. We have also carried out calculations by using the hydroxyl binding formulation in addition to our proton binding method and verified the results to be identical. Table I gives a summary of values estimated for analysis of the Roughton-Lyster data. These results correspond to the results presented by Szabo & Karplus (1972) (fits (ii)<sub>1</sub> and (ii)<sub>2</sub> of their Table I), where the energetic terms for Bohr protons are held fixed, and the salt bridge energies are also set at 2.00 kcal for each of the three types of salt bridge.

Our program provides several additional kinds of information not explicitly calculated by Szabo and Karplus: (a) The



Table I: Analysis of Roughton & Lyster Data (1965) for HbA with Extended Szabo-Karplus Model<sup>a</sup>

parameter	pH 7.0	pH 9.1
$\delta_{4\alpha}$	-8.66 (-8.91, -8.40)	-8.66 (-8.93, -8.35)
$\delta_{4\beta}$	-8.26 (-8.43, -8.06)	-8.38 (-8.61, -8.13)
$\delta_S^c$	-2.00 <sup>c</sup>	-2.00 <sup>c</sup>
$\delta_{\alpha}^H$	-10.39 <sup>b</sup>	-10.39 <sup>b</sup>
$\delta_{\beta}^H$	-7.99 <sup>b</sup>	-7.99 <sup>b</sup>
$\delta_Q$	-5.08 (-5.39, -4.85)	-5.73 (-6.00, -5.48)
$\bar{\nu}_{40}^{H^+}$	0.877 (0.877, 0.877)	0.318 (0.317, 0.318)
$\bar{\nu}_{44}^{H^+}$	0.477 (0.477, 0.477)	0.242 (0.242, 0.242)
$f_{\alpha}^d$	0.21, 0.87	0.44, 0.62
variance of fit	$2.09 \times 10^{-5}$	$4.34 \times 10^{-5}$

<sup>a</sup> The experimental oxygen binding curves were fit for the tetrameric parameters of the Szabo-Karplus model under the same assumptions used by them, except for the difference in formulation of the Bohr proton reactions (see text). The values listed here correspond to the fits listed in their Table I: fit (ii)<sub>1</sub> for pH 7 and fit (ii)<sub>2</sub> for pH 9.1. Confidence limits corresponding to 65% probability are given in parentheses. The protein concentration pertaining to these data was sufficiently high that dimeric species need not be considered. Our computer program, however, requires values for the properties of the dimeric species. Consequently we used our "unconstrained" model with  ${}^0\Delta G_2 = -14.38$ ,  $K_{2\alpha} = K_{4\alpha}$ , and  $K_{2\beta} = K_{4\beta}$ . Henry's law constant was taken to be  $1.78 \times 10^{-6}$  M/mmHg, and the temperature was 19 °C. <sup>b</sup> Values fixed for the same  $pK_a$ s assumed by Szabo & Karplus (1972). <sup>c</sup> It was assumed that the salt bridges were identical as per Szabo & Karplus (1972), i.e.,  $\delta_s = \delta_{\alpha\alpha} = \delta_{\alpha\beta} = \delta_{\beta\beta}$ . <sup>d</sup> The fraction of oxygens on the  $\alpha$  chains of single- and triple-liganded hemoglobin (see eq 23 and text).

confidence limits provide error estimates for the fitted model parameters. (b) The number of Bohr protons bound for deoxy and oxy tetramers is shown in Table I. The difference between these numbers yields the number of Bohr protons released upon oxygenation. At pH 7, a Bohr effect of 0.4 proton/mol of heme was estimated, whereas the value at pH 9.1 is decreased to 0.18 proton/mol of heme. These values are generally reasonable for the known alkaline Bohr effect. Although the value at pH 7.0 appears somewhat low compared with reported values (cf. Antonini et al., 1965), the value has not been determined experimentally under the high phosphate conditions of Roughton and Lyster's oxygenation data. (c) Our program also calculates the distribution of oxygen molecules bound to the  $\alpha$  and  $\beta$  chains of hemoglobin tetramers at the various states of oxygenation. It is of particular interest to examine the fraction,  $f_{\alpha}$ , of oxygens bound to the  $\alpha$  chains on singly oxygenated tetramers and on triply liganded tetramers. From the fits of the model to the data, the distribution of oxygens was found to be highly unequal. At pH 7.0, 21% of the oxygens on singly oxygenated tetramers are bound to the  $\alpha$  chains, whereas for triply liganded tetramers, 87% of the oxygens are bound to  $\alpha$  chains. At pH 9.1, the corresponding percentages bound to  $\alpha$  chains of singly and triply oxygenated tetramers are 44% and 62%, respectively. This result of the Szabo-Karplus model for tetramers is inconsistent with the experimental results of Johnson & Ho (1974) and Viggiano & Ho (1979) which show by nuclear magnetic resonance a preference for the  $\alpha$  chain in the presence of organic phosphate and no preferential binding to either chain in the absence of organic phosphate (Johnson & Ho, 1974; Viggiano & Ho, 1979). Although the relative chain affinities have not been determined in 0.15 M inorganic phosphate, the NMR results cited above suggest that the preferential binding to chains is unlikely.

Table II: Model-Independent Thermodynamic Properties for the Linkage System in Hemoglobins A and Kansas

free energy	hemoglobin A <sup>a</sup>	hemoglobin Kansas <sup>b</sup>
$\Delta G_{21}^c$	-8.38 ± 0.2	-8.40
$\Delta G_{22}^c$	-8.38 ± 0.2	-7.34
$\Delta G_{41}^d$	-5.45 ± 0.2	-5.81 ± 0.1
$\Delta G_{42}^d$	-5.28 ± 0.5	-5.41 ± 0.4
$\Delta G_{43}^d$	-7.80 ± 0.6	-5.59 ± 0.5
$\Delta G_{44}^d$	-8.65 ± 0.4	-6.36 ± 0.4
${}^0\Delta G_2^e$	-14.38 ± 0.2	-13.63 ± 0.2
${}^1\Delta G_2^e$	-11.46 ± 0.3	-11.41 ± 0.2
${}^2\Delta G_2^e$	-7.78 ± 0.2	-7.48 ± 0.2
${}^4\Delta G_2^e$	-8.05 ± 0.2	-5.29 ± 0.2

<sup>a</sup> Data analyzed are those of Mills et al. (1976) and Ip & Ackers (1977). Conditions: 0.1 M Tris-HCl, 0.1 M NaCl, pH 7.4, and 1 mM Na<sub>2</sub>EDTA, 21.5 °C. Values are taken from Mills et al. (1976); variance of fit =  $2.57 \times 10^{-5}$ . <sup>b</sup> Data of Atha et al. (1979). Conditions: 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.5, and 1 mM Na<sub>2</sub>EDTA, 20.0 °C. Variance of fit =  $1.3 \times 10^{-4}$ . <sup>c</sup> Free energies for sequential binding reactions of oxygen by dimers, corrected for statistical factors. <sup>d</sup> Free energies for sequential binding reactions of oxygen by tetramers, corrected for statistical factors. <sup>e</sup> Free energy of formation of tetramers  ${}^i\Delta G_2$  with  $i$  oxygens bound, corrected for statistical factors.

In the original Szabo-Karplus model, the values of salt bridge energies are constrained to be identical. We have relaxed this constraint and extended the model to include independent variations in the three salt bridge energies. Results of fitting the Roughton pH 7.0 data to this more general model yielded values of  $\delta_{\alpha\alpha} = -2.00$  kcal,  $\delta_{\alpha\beta} = -1.99$  kcal, and  $\delta_{\beta\beta} = -1.98$  kcal with the same values of all other parameters as shown in Table I. We next imposed the constraint of requiring  $f_{\alpha}$  to equal 0.5 for both singly and triply liganded species, as formulated in eq 23, while simultaneously allowing values of the salt bridge energies to float. With this constraint, we were unable to find any fit with reasonable variance to the Roughton-Lyster data for any values of the salt bridge energies.

(B) *Data of Mills et al. (1976).* The hemoglobin A<sub>0</sub> data of Mills et al. (1976) for normal human hemoglobin were the first data which permitted a complete resolution of the thermodynamic parameters for the linkage between subunit association ( $2_{\alpha\beta} \leftrightarrow \alpha_2\beta_2$ ) and oxygen binding (mechanism 1). It consists of a series of oxygen binding curves each measured at a fixed total hemoglobin concentration which varied by approximately 2 orders of magnitude ( $4 \times 10^{-4}$  to  $5 \times 10^{-6}$  M heme). The curves are shown in Figure 2. These data were combined with independently measured values for the subunit association constants for formation of fully oxygenated ( ${}^4K_2$ ) and fully deoxygenated ( ${}^0K_2$ ) tetramers (Ip et al., 1976; Ip & Ackers, 1977) in order to resolve completely all of the equilibria depicted in mechanism 1. The model-independent thermodynamic results from analysis of these data are listed in Table II. We used these data to evaluate the statistical thermodynamic model parameters at 21.5 °C in 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM Na<sub>2</sub>EDTA, pH 7.4. Two separate versions of the model were employed.

*Dimer with No Constraints.* For evaluation of the simplest model where the dimeric species have no constraints, the intrinsic free energy for assembly of the deoxygenated tetramer ( ${}^0\Delta G_2$ ) and the oxygen binding energies for the dimer ( $\Delta G_{21}$  and  $\Delta G_{22}$ ) were taken to have the values found in the model-independent analysis of these data (Mills et al., 1976). As reported by Szabo & Karplus (1972), we also found that the remaining eight parameters of the model were highly correlated with each other and could not all be simultaneously evaluated. Therefore the proton binding free energies  $\delta_{\alpha}^H$  and



Table III: Analysis of Data of Mills et al. (1976) for HbA According to the Extended Szabo-Karplus Model: Unconstrained Dimer<sup>a</sup>

	case A	case B
$^0\Delta G_2$	-14.38 <sup>b</sup>	-14.38 <sup>b</sup>
$\delta_{2\alpha}$	-8.38 <sup>b</sup>	-8.38 <sup>b</sup>
$\delta_{2\beta}$	-8.38 <sup>b</sup>	-8.38 <sup>b</sup>
$\delta_{4\alpha}$	-11.26 (-11.46, -11.09)	-14.48 (-14.65, -14.32)
$\delta_{4\beta}$	-9.53 (-9.77, -9.27)	-14.52 (-14.74, -14.24)
$\delta_{\alpha\alpha}$	-2.96 (-3.08, -2.86)	-5.35 (-5.50, -5.22)
$\delta_{\alpha\beta}$	-3.07 (-3.30, -2.82)	-2.53 (-2.74, -2.37)
$\delta_{\beta}$	-2.98 (-3.18, -2.76)	-8.08 (-8.50, -7.54)
$\delta_{\alpha}^H$	-10.39 <sup>b</sup>	-10.39 <sup>b</sup>
$\delta_{\beta}^H$	-7.99 <sup>b</sup>	-7.99 <sup>b</sup>
$\delta_Q$	-5.34 (-5.61, -5.15)	-7.44 (-7.77, -7.15)
$\bar{\nu}_{40}^{H^+}$	0.925 (0.899, 0.944)	1.000 (1.000, 1.000)
$\bar{\nu}_{44}^{H^+}$	0.358 (0.358, 0.358)	0.358 (0.358, 0.358)
$\bar{\nu}_{20}^{H^+}$	0	0
$\bar{\nu}_{22}^{H^+}$	0	0
$f_{\alpha}^c$	0.58, 0.97	0.04, 0.99
variance of fit	$2.60 \times 10^{-5}$	$2.59 \times 10^{-5}$

<sup>a</sup> Analysis of data of Mills et al. (1976) according to the Szabo-Karplus model extended to include dimers with no constraints and with a modified formulation of the Bohr proton reactions.

<sup>b</sup> Assumed values; see text for details. <sup>c</sup> The fraction of oxygens on the  $\alpha$  chains of single- and triple-liganded hemoglobin (see eq 23 and text).

$\delta_{\beta}^H$  were fixed at various combinations of values which yielded reasonable values for the tetramer Bohr effect, and the remaining parameters were allowed to float. We describe here only the results of the fits which were the most successful in terms of yielding the most reasonable sets of parameters. For these fits,  $\delta_{\alpha}^H$  and  $\delta_{\beta}^H$  have the same values as shown in Table I for the analysis of the Roughton-Lyster data. Table III presents two analyses of the data of Mills et al., for the remaining six parameters (cases A and B, Table III). Also given in Table III are the number of protons bound to the tetramers in deoxy and oxy states and the fraction,  $f_{\alpha}$ , of oxygens which bind to  $\alpha$  chains at the first and third oxygenation step.

Comparison of cases A and B (Table III) reveals the problem encountered with local minima. Values obtained for the model parameters in the two fits are seen to be significantly different. However, the sets of model parameters for both of the fits gave essentially identical thermodynamic constants  $K_m$ , and these in turn fall within the confidence limits of the model-independent analyses (see Table II for a listing of the model-independent thermodynamic values). Consequently, the least-squares fits obtained with this model are not unique and depend upon the region of the multidimensional space taken for the initial guesses of the iterative least-squares algorithm.

Case B is not a realistic minimum. The values for the free energies of two of the salt bridges ( $\alpha$  and  $\beta$ ) are far outside the range of physically expected values. Additionally it yields values for the intrinsic affinities of the chains,  $\delta_{4\alpha}$  and  $\delta_{4\beta}$ , within tetramers which are unreasonably large. From the standpoint of these considerations, case A is the more realistic solution. However, the values of intrinsic binding energies for oxygen,  $\delta_{4\alpha}$  and  $\delta_{4\beta}$ , appear unexpectedly large in this fit as well. The value of  $\delta_{4\beta}$  corresponds to a binding constant of  $2.3 \times 10^8 \text{ M}^{-1}$  whereas the affinities of isolated chains measured under identical conditions are 2 orders of magnitude lower (Mills & Ackers, 1979). Thus the value of  $\delta_{4\beta}$  appears to be considerably outside the range of physically meaningful values.

The distribution of oxygens on singly liganded tetramers as predicted from the least-squares fitted parameters is quite different for the two fits. Case A predicts that the oxygen is on an  $\alpha$  chain in 58% of the singly liganded tetramers and 99% of the triply liganded molecules. Case B predicts that only 4% of the oxygens are on the  $\alpha$  chains for singly liganded tetramers. The conditions under which the data of Mills et al. were obtained are very close to those of the nuclear magnetic resonance results (Johnson & Ho, 1974; Viggiano & Ho, 1979) which indicate no preferential binding to either chain to within a maximum difference of approximately 10%. This extrathermodynamic experimental result along with unexpectedly large values of  $\delta_{4\alpha}$  and  $\delta_{4\beta}$  casts serious doubt on the validity of the model.

Efforts to constrain  $\delta_{4\alpha}$  and  $\delta_{4\beta}$  to values similar to the intrinsic affinities of the isolated chains (Mills & Ackers, 1979) while simultaneously constraining  $f_{\alpha}$  to 0.5 resulted in poorer fits and clearly impossible values of the tetramer Bohr effect. For example, it was possible to fit the data with  $\delta_{4\alpha} = -8.47 \text{ kcal}$ ,  $\delta_{4\beta} = -8.74 \text{ kcal}$ ,  $\delta_{\alpha\alpha} = -2.96 \text{ kcal}$ ,  $\delta_{\alpha\beta} = -3.07 \text{ kcal}$ ,  $\delta_{\beta} = -2.98 \text{ kcal}$ ,  $\delta_{\alpha}^H = -6.07 \text{ kcal}$ ,  $\delta_{\beta}^H = +2.83 \text{ kcal}$ , and  $\delta_Q = -5.52 \text{ kcal}$ . In this fit (variance  $3.9 \times 10^{-5}$ ) the tetramer Bohr effect was predicted to be 0.06, which is less than one-eighth of its correct value.

Following the original proposal of the Perutz mechanism (1970a,b), a series of experimental studies have been conducted to assess the contributions of various amino acid residues to the alkaline Bohr effect (cf. Kilmartin & Hewitt, 1971; Kilmartin & Rossi-Bernardi, 1971; Kilmartin et al., 1973, 1980; Perutz et al., 1980). The results of these studies were interpreted to indicate that only about 70% of the alkaline Bohr effect could be attributed to the residues  $\alpha 1 \text{ Val}$  and  $\beta 146 \text{ His}$ . We were therefore interested in knowing whether adjustment of the Bohr effect parameters  $\delta_{\beta}^H$  and  $\delta_{\alpha}^H$  to reduce the calculated Bohr effect by 30% would lead to more reasonable values of the intrinsic binding energies. It was found that no such adjustment could significantly improve upon the physically untenable values of  $\delta_{4\alpha}$  shown in Tables III-V.

(C) *Hemoglobin Kansas*. The variant human hemoglobin Kansas is of particular interest in studies of ligand-linked dimer-tetramer assembly because the amino acid substitution ( $\alpha 102 \text{ asparagine} \rightarrow \text{threonine}$ ) is in the contact region between the  $\alpha_1\beta_1$  dimers. The X-ray structural analysis of hemoglobin Kansas indicated a hydrogen bond in the  $\alpha_1\beta_2$  contact ( $\alpha 99 \text{ Asn}-\beta 102 \text{ Gln}$ ), which is present in oxy-hemoglobin A but not in oxygenated Kansas, whereas their deoxy structures are thought to be identical (Greer, 1971; Anderson, 1975). Atha et al. (1979) have published oxygen binding curves for Hb Kansas at a series of hemoglobin concentrations. They combined these measurements with an earlier determination of the dimer-tetramer association constant (Atha & Riggs, 1976) to obtain a complete thermodynamic description of hemoglobin Kansas in terms of the parameters in mechanism 1. The structural alteration in hemoglobin Kansas has little effect on the subunit association constant of unliganded hemoglobin Kansas. But in the oxygenated molecule, the difference in association constants between Kansas and normal hemoglobins is large. A summary of the model-independent thermodynamic constants for Hb Kansas is given in Table II. We also note that hemoglobin Kansas has a normal Bohr effect. Szabo & Karplus (1972) predicted that the energy of quaternary structure change,  $\delta_Q$ , for hemoglobin Kansas would be different from the values found for hemoglobin A by several orders of magnitude. However, the free energies for forming the salt bridges ( $\delta_{\alpha\alpha}$ ,

Table IV: Analysis of Data of Mills et al. (1976) for HbA According to the Extended Szabo-Karplus Model: Constrained Dimer<sup>a</sup>

$^0\Delta G_2$	-14.38 <sup>b</sup>
$\delta_{2\alpha}$	-8.38 (-8.77, -7.98)
$\delta_{2\beta}$	-9.51 (-9.91, -9.10)
$\delta_{4\alpha}$	-11.28 (-11.34, -11.25)
$\delta_{4\beta}$	-9.53 (-9.65, -9.45)
$\delta_{\alpha\alpha}$	-3.01 (-3.05, -2.97)
$\delta_{\alpha\beta}$	-2.96 (-3.15, -2.84)
$\delta_{\beta\beta}$	-3.02 (-3.09, -2.96)
$\delta_{\alpha}^H$	-10.39 <sup>b</sup>
$\delta_{\beta}^H$	-7.99 <sup>b</sup>
$\delta_Q$	-5.36 (-5.39, -5.33)
$\bar{\nu}_{40}^H$	0.939 (0.923, 0.936)
$\bar{\nu}_{44}^H$	0.358 (0.358, 0.358)
$\bar{\nu}_{30}^H$	0.772 (0.766, 0.779)
$\bar{\nu}_{22}^H$	0.385 (0.358, 0.358)
$f_{\alpha}^c$	0.52, 0.98
variance of fit	$2.59 \times 10^{-5}$

<sup>a</sup> Szabo-Karplus model was extended to include the effects of dimers with constraints and with a modified formulation of the Bohr proton reactions (see text for details). <sup>b</sup> Assumed values; see text for details. <sup>c</sup> The fraction of oxygens on the  $\alpha$  chains in single- and triple-liganded hemoglobin (see eq 23 and text).

$\delta_{\alpha\beta}$  and  $\delta_{\beta\beta}$  were not expected to be significantly different since the mutation is believed not to alter these interactions. The mutation is known, however, to alter significantly the affinity of the isolated chain; i.e., the Kansas  $\beta$  chain has much lower affinity for oxygen than the HbA  $\beta$  chain (Riggs & Gibson, 1973). Consequently, the intrinsic affinities of the  $\beta$  chains within the tetramer may also be expected to differ from the normal hemoglobin case.

For analysis of the data of Atha et al. in terms of the Szabo-Karplus model, a similar procedure was used to that of the hemoglobin A analyses. In the best fits obtained, the proton binding energies,  $\delta_{\alpha}^H$  and  $\delta_{\beta}^H$ , were fixed at -10.39 and -7.99 kcal/mol, respectively. The subunit association energy of deoxyhemoglobin Kansas ( $K_2$ ) was fixed at the independently determined value of -13.63 kcal/mol (Atha et al., 1979). For the model with no constraints on the dimer, the values of the dimeric binding free energies,  $\Delta G_{21}$  and  $\Delta G_{22}$ , were taken to be -8.27 and -7.47 kcal/mol, respectively (Atha et al., 1979).

Results of the analysis of the hemoglobin Kansas data are given in Table V. As in the case of hemoglobin A, it was found that both versions of the extended Szabo-Karplus model were capable of fitting the experimental data with the same precision as the model-independent thermodynamic fits (not shown). The two models differ considerably in the value of  $\delta_{2\beta}$  from the models for HbA (Tables III and IV). The magnitude of  $\delta_{4\alpha}$  for Hb Kansas (Table V) is far outside the range of expected values. A further comparison of results for hemoglobins A and Kansas (Tables III-V) shows very similar energies for oxygenation of the dimeric chains, for the tetrameric oxygen affinities, and for the salt bridges. The value of  $\delta_Q$  is essentially undefined by the Hb Kansas data, as indicated by the enormously broad confidence limits. The value converged on for  $\delta_Q$  is similar to that for HbA. Thus the major difference in parameters between hemoglobins A and Kansas for the constrained dimer model is in the magnitudes of the chain affinities, particularly that of the  $\beta$  chain,  $\delta_{4\beta}$ , whereas the value of the quaternary structure parameter,  $\delta_Q$ , does not appear to differ greatly from its value in hemoglobin A. For both versions of the model, the predicted Bohr effect is about 0.65 proton/mol of oxygen. This value is slightly larger than that

Table V: Analysis of Hemoglobin Kansas Data of Atha et al. (1979) According to the Extended Szabo-Karplus Model

	(A) unconstrained dimer	(B) constrained dimer
$^0\Delta G_2$	-13.63 <sup>a</sup>	-13.63 <sup>a</sup>
$\delta_{2\alpha}$	-8.27 <sup>a</sup>	-8.36 (-8.70, -8.05)
$\delta_{2\beta}$	-7.47 <sup>a</sup>	-9.74 (-10.70, -8.94)
$\delta_{4\alpha}$	-13.62 (-15.33, -13.03)	-13.86 (-14.91, -13.06)
$\delta_{4\beta}$	-8.30 (-9.30, -7.91)	-8.51 (-9.23, -7.98)
$\delta_{\alpha\alpha}$	-3.86 (-4.71, -3.57)	-3.98 (-4.50, -3.59)
$\delta_{\alpha\beta}$	-3.88 (-14.10, +0.67)	-4.64 (-10.43, +1.52)
$\delta_{\beta\beta}$	-4.17 (-5.34, -3.76)	-4.41 (-5.16, -3.84)
$\delta_{\alpha}^H$	-10.39 <sup>a</sup>	-10.39 <sup>a</sup>
$\delta_{\beta}^H$	-7.99 <sup>a</sup>	-7.99 <sup>a</sup>
$\delta_Q$	-6.28 (-27.00, +3.08)	-7.88 (-19.48, +4.48)
$\bar{\nu}_{40}^H$	0.987 (0.975, 0.998)	0.991 (0.978, 0.998)
$\bar{\nu}_{44}^H$	0.340 (0.340, 0.340)	0.340 (0.340, 0.340)
$\bar{\nu}_{30}^H$	0	0.818 (0.804, 0.824)
$\bar{\nu}_{22}^H$	0	0.340 (0.310, 0.340)
$f_{\alpha}$	0.99	0.99
variance of fit	$1.38 \times 10^{-4}$	$1.35 \times 10^{-4}$

<sup>a</sup> Assumed values.

expected. The value of  $f_{\alpha}$  indicates that 99% of the first oxygens bound occupy the  $\alpha$  chains. This is presumably a result of the enormously high values of  $\delta_{4\alpha}$ . In general the values of salt bridge energies are also estimated to be higher in Hb Kansas than in HbA.

## Discussion

In this study we have used the statistical thermodynamic model of Szabo and Karplus to explore the relationship between the Perutz mechanism and recent thermodynamic information on human hemoglobins. In order to do so, we have extended the model to include the effects of tetrameric dissociation along the  $\alpha_1\beta_2$  contact into  $\alpha_1\beta_1$  dimers. This new dimension of analysis is of particular interest since the major ligand-linked structure changes within tetrameric molecules are known to occur at the  $\alpha_1\beta_2$  interface. In one version (constrained dimers), we have preserved as many properties as is physically reasonable for the dimer under the original Szabo-Karplus rules. With this model and also with a contrasting set of assumptions regarding the properties of dissociated dimers (the unconstrained model), we have tested the extended models against recent experimental results on the ligand-linked subunit assembly of human hemoglobins A and Kansas. From results of these analyses, we have found that both of the extended models are mathematically capable of describing the recent experimental data on oxygenation-linked subunit association for both hemoglobins A and Kansas. In addition the parameters which are expected on model-independent grounds to be the same between hemoglobins A and Kansas are predicted by the model to have similar values.

In spite of this consistency, the actual values of several key parameters predicted by the model do not appear physically reasonable. The model was found incapable of simultaneously predicting correct values for the tetramer Bohr effect and physically reasonable values of the intrinsic chain binding constants. These difficulties were compounded further when the requirement of equal chain affinities was imposed. Under this criterion, no successful fits were obtained for the Roughton-Lyster data; for the Mills data, the tetramer Bohr effect was predicted to be about one-ninth of its correct value when other parameters were forced into physically reasonable ranges. These general characteristics of the properties for tetrameric hemoglobin were essentially independent of the properties assumed for the dimers. For all fits in which the

requirement of equal chain binding probabilities was removed, the fits predicted highly unequal distributions of bound oxygen molecules of the intermediate states of ligation. This prediction is inconsistent with the independent experimental findings of equal chain affinities under these conditions.

We conclude that the Perutz/Szabo-Karplus model is incapable of accommodating the composite body of highly reliable experimental information now available on the hemoglobin system. This conclusion of course applies only to the specific formulation of the Perutz mechanism embodied in the Szabo-Karplus model. It should be emphasized that the inconsistencies found in this study between the extended Szabo-Karplus model and the thermodynamic data do not constitute unequivocal proof against the Perutz mechanism. As noted earlier, the statistical thermodynamic model is not a perfect translation of the Perutz proposals. We thus cannot say that no alternative formulation of the Perutz mechanism could be found consistent with the data.

A central question to this work then is to what extent does quantitative disagreement between such a model and experimental data invalidate the qualitative ideas upon which the model is based? In order to answer this question, it is important to consider the fundamental reasons for failure of the model. It is clear that failure of the Szabo-Karplus model is not due to any shortage of molecular states, but rather it fails as a result of the stringency of constraints imposed by the set of rules upon which it is based. Clearly other sets of rules which lead to different probabilities for the states of the system might accommodate the data better. How much modification of the rules would be required to overcome the difficulties encountered by the presently existing model is impossible to determine simply and is beyond the scope of this work. Thus we can only conclude that failure of the existing model provides evidence against the Perutz mechanism in general and particularly against the specific rules which define, at a detailed structural level, the relationships between various processes within the molecular system.

Additional insight arises from considering the relationship of the work described in this paper to recent findings which have a bearing on the validity of the general ideas underlying the Perutz mechanism:

(a) *Role of Salt Bridges.* The structural configuration of pairwise interactions in the Szabo-Karplus model simulate the topological pattern of oxygenation-sensitive salt bridges found in the actual structure of hemoglobin. Apart from this, the mathematical model does not distinguish between pairwise interactions as to their chemical type. As long as the topology is the same, the bonds which stabilize the deoxy quaternary structure and oppose oxygenation might be of any chemical type. Failure of the model is therefore primarily a failure of the rules used to alter the interactions upon oxygenation rather than any evidence against the salt bridges as the dominant stabilizing factor. However, strong evidence against the salt bridges as providing the dominant source of energy for stabilization of the deoxy form of hemoglobin (vs. oxy) comes from the following recent work: (1) The model independent thermodynamic data for human hemoglobin have recently been analyzed for consistency with various types of noncovalent interactions representing possible sources of cooperative energy (Ackers, 1980). The patterns of thermodynamic effects were found to provide evidence against a dominant role of salt bridges in stabilizing the deoxy quaternary structure. The thermodynamic information is consistent with dominance of hydrogen bonding and van der Waals interactions. (2) Recent results indicating insensitivity of the dimer-tetramer associ-

ation constant of deoxy hemoglobin to salt [Chu & Ackers, 1981; see also Thomas & Edelstein (1973)] are consistent with this conclusion. (3) Recent calculations of Flanagan et al. (1981) have evaluated the contribution of electrostatic interactions to the free energy of stabilization of the dimer-dimer interface in deoxy- and oxyhemoglobins. Results of these analyses indicate a greater electrostatic stabilization of oxyhemoglobin as compared to the unliganded molecule. These results once again argue against a dominant role of salt bridges in accounting for the additional stabilization free energy of the deoxy quaternary structure.

(b) *Tetramer Bohr Effect.* The Szabo-Karplus model is based upon a treatment of the tetramer Bohr effect in terms of only two ionizing groups, one of which corresponds to the  $\beta$ 146 histidine residue. Recent nuclear magnetic resonance studies (Russu et al., 1980) have shown this group to contribute significantly to the Bohr effect in the presence of 0.2 M phosphate and 0.2 M chloride, corresponding more closely to conditions of the Roughton-Lyster data. But the  $\beta$ 146 histidine contributes only negligibly to the Bohr effect on 0.5 M Bistris buffer under conditions more similar to that of the data sets obtained by Mills et al. (1976) and by Atha et al. (1979).

Two additional problems regarding treatment of the Bohr effect in the Szabo-Karplus model should be noted. First, the model assumes no change in  $pK_a$  values for the ionizable groups, whereas the NMR studies have unequivocally established that significant  $pK$  shifts do occur (cf. Russu et al., 1980; Gurd et al., 1980). Second, it is likely that a large number of groups contribute significantly to the Bohr effect. Electrostatic calculations by Matthew et al. (1979) indicate that as many as 11 groups may contribute appreciably under some conditions. Arguments against these findings of Russu et al. (1980) and of Matthew et al. (1979) with regard to the nature of the Bohr effect (Kilmartin et al., 1980) do not appear convincing in our view.

It should be noted that as a result of recent experimental studies, much greater demand is now placed on any detailed model of hemoglobin function. The analysis of thermodynamic results, described in this paper, provide evidence against certain of the ideas of the Perutz mechanism, particularly those regarding the role of salt bridges and Bohr protons. Taken together with the other evidence cited above, it would appear desirable to consider alternative theories of the hemoglobin mechanism. The statistical thermodynamic approach to this problem pioneered by Szabo & Karplus (1972) appears to offer great promise in this regard since it provides a means of accounting for the pairwise interactions between parts of the hemoglobin molecules at any desired level of detail and provides a powerful means of correlating the energetic and structural properties of the system.

#### Supplementary Material Available

Tables showing the distribution of constraints in tetrameric hemoglobin according to the model of Szabo-Karplus (Appendix A), the oxygenation data of hemoglobin A from Mills et al. (1976) (Appendix B), and the oxygenation data of hemoglobin Kansas from Atha et al. (1979) (Appendix C) (19 pages). Ordering information is given on any current masthead page.

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