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Analysis of the Order of Free Energy Couplings between Ligand Binding and Subunit Assembly in Human Hemoglobin†

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ABSTRACT: The concept of free energy couplings has been extensively used in studies of the ligand-linked subunit assembly of oligomeric proteins such as human hemoglobin A [cf. Ackers, G. K. (1980) *Biophys. J. 32*, 331–346]. Recently, the concept of "order" of free energy couplings has been introduced as a description of the number of protein subunits that must be liganded to effect changes in intersubunit interactions [Weber, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7098–7102]. That report utilized the concept of order of free energy couplings to analyze a set of previously published equilibrium constants derived from data pertaining to the chemical equilibrium between oxygen and stripped hemoglobin A [Mills, F. C., Johnson, M. L., & Ackers, G. K. (1976) *Biochemistry 15*, 5350–5362]. The Weber report claims to have "unequivocally" demonstrated that the coupling between oxygenation and subunit assembly in hemoglobin A is "first order". In the present report, it is demonstrated that free energy couplings of both the first and second order are capable of describing the original oxygen binding data.

A large amount of information regarding structural changes within human hemoglobin that accompany oxygenation has been provided by X-ray crystallographic studies [cf. Baldwin & Chothia (1979) and Perutz et al. (1969)] as well as X-ray absorption fine structure spectroscopy (Eisenberger et al., 1978), resonance Raman spectroscopy (Asher et al., 1981), and NMR spectroscopy (Russu et al., 1983; Viggiano & Ho, 1979; Viggiano et al., 1979). In addition, ligand binding studies have provided a knowledge of the free energy changes that are concomitant with the oxygenation–deoxygenation cycle [cf. Ackers (1980), Ackers & Halvorson (1974), Ackers & Johnson (1981), Chu et al. (1984), Flanagan et al. (1981), Johnson & Ackers (1982), Mills & Ackers (1979), Mills et al. (1976), Pettigrew et al. (1982), and Smith & Ackers (1985)]. The combination of these findings imposes stringent constraints on the nature of the structural and thermodynamic interactions responsible for cooperativity in human hemoglobin.

A number of "models" of cooperativity of oxygen binding to hemoglobin have appeared in the literature [cf. Ackers & Johnson (1981), Herzfeld & Stanley (1974), Johnson & Ackers (1982), Johnson et al. (1984), Koshland et al. (1966), Lee & Karplus (1983), Monod et al. (1965), Perutz (1970a,b), Szabo & Karplus (1972), and Weber (1972, 1982)]. Some of these models have been explicitly formulated to include the linkage between subunit assembly and oxygen binding (Ackers & Johnson, 1981; Johnson & Ackers, 1982; Johnson et al., 1984). Recently, Weber (1984) claimed to have demonstrated that the concept of a first-order free energy coupling[1] is necessary and sufficient to describe the linkage between subunit assembly and oxygen binding in human hemoglobin A. However, a number of the literature models are capable of predicting the same equilibrium constants that Weber used for his demonstration and at the same time are inconsistent with the concept of first-order free energy coupling (Ackers & Johnson, 1981; Johnson & Ackers, 1982; Johnson et al., 1984; Lee & Karplus, 1983). The purpose of this report is to demonstrate that second-order free energy coupling is also capable of describing the original oxygen binding data. The oxygen binding data employed for this demonstration are the same data that were used to evaluate the equilibrium constants (Mills et al., 1976) that Weber (1984) subsequently used to assert that free energy coupling in human hemoglobin is first order.

Experimental Data Employed

Two independent sets of previously published data were used in this study (Chu et al., 1984; Mills et al., 1976). Both sets represent the binding of oxygen to stripped human hemoglobin A as a function of both hemoglobin concentration and oxygen concentration. In addition to the oxygen binding data, each of these sets of data includes an independent evaluation of the dimer to tetramer association constant for both oxygenated and deoxygenated hemoglobin. The actual experimental conditions for both data sets were 0.1 M tris(hydroxymethyl)aminomethane (Tris), 0.1 M NaCl, and 1.0 mM disodium ethylenediaminetetraacetate (Na_2EDTA), titrated to pH 7.4 with concentrated HCl, at 21.5 °C. The "Mills et al. data" were used to evaluate the equilibrium constants that Weber subsequently used to describe the order of free energy couplings. The other data set will be referred to as the "Chu et al. data".

In both of these sets of data the binding of oxygen was measured as a function of oxygen concentration at a range of hemoglobin concentrations. For example, the range of protein concentration in the Chu et al. data was from 4.5 to 382 μM heme. It has previously been shown that at these low concentrations of hemoglobin a significant fraction of the oxygenated hemoglobin will occur as dimers while the deoxygenated hemoglobin will exist predominantly as tetramers (Ackers & Halvorson, 1974; Johnson & Ackers, 1977; Johnson

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[1] The order of free energy coupling refers to the number of hemoglobin subunits that must be liganded in order to induce an alteration in the free energy of interaction between the specific subunits. It does not refer to a specific sequence of adding ligands to the hemoglobin tetramer. See Order of Free Energy Couplings Concept for a more complete description.

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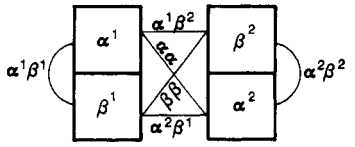


FIGURE 1: Geometry of the hemoglobin tetramer molecule with individual α and β subunits labeled. Also shown are all possible intersubunit interactions allowed by first- and second-order free energy couplings: $\alpha^i\beta^i$, $\alpha^i\beta^j$, $\alpha\alpha$, and $\beta\beta$.

et al., 1976; Mills et al., 1976; Valdes & Ackers, 1978). The purpose in doing the experiments at these low concentrations was to utilize the hemoglobin concentration dependence as an additional method of probing the intersubunit interactions which are altered upon oxygenation. A consequence of this is that the dimer–tetramer equilibrium and the binding of oxygen to the dimers must be included in any method of analysis used to evaluate the oxygen binding properties of the tetramers from these data sets (Johnson & Ackers, 1977). This is of particular importance for mutant hemoglobins, such as hemoglobin Kansas for which as much as 7% of the hemoglobin exists as dimers at physiological concentrations within a red blood cell (Atha et al., 1979).

For the analysis presented here, the original oxygen binding data were used rather than equilibrium constants derived from the data, or synthetic data generated from the derived equilibrium constants. This use of the original data is required since it allows the least-squares analysis method direct access to the actual experimental observations and their concomitant experimental uncertainties. This permits a comparison of different mechanisms of oxygen binding and different relative minima of the same mechanism, based on changes in the variance of the least-squares analysis.

Order of Free Energy Couplings Concept

From X-ray crystallographic studies (Baldwin & Chothia, 1979; Perutz et al., 1969) it is known that upon oxygenation the hemoglobin tetramer undergoes a structural isomerization along an interface between two identical $\alpha\beta$ dimers. These dimers are referred to as $\alpha^1\beta^1$ and $\alpha^2\beta^2$. This same nomenclature will be used to denote the individual α and β subunits in describing the concept of the order of free energy couplings (Weber, 1984).

The most general case of the first- and second-order free energy couplings concept applied to hemoglobin incorporates 10 distinct parameters. Three of these describe the oxygen binding properties of the dimeric oligomer, six describe the oxygen binding properties of the tetrameric oligomer, and one refers to the self-association of the dimers to form tetramers. In this report, a number of assumptions are made to decrease the total number of unknown parameters. These are the same assumptions that were utilized in the original description of the concept of order of free energy couplings in human hemoglobin (Weber, 1984).

In the free energy couplings concept (Weber, 1984) of the hemoglobin tetrameter there are six possible oxygenation-sensitive[2] free energies of interaction between the following pairs of subunits: $\alpha^1\beta^1$, $\alpha^2\beta^2$, $\alpha^1\beta^2$, $\alpha^2\beta^1$, $\alpha\alpha$, and $\beta\beta$. $\{\alpha^1\beta^1\}$, $\{\alpha^2\beta^2\}$, $\{\alpha^1\beta^2\}$, $\{\alpha^2\beta^1\}$, $\{\alpha\alpha\}$, and $\{\beta\beta\}$ designate the differences between unliganded and liganded free energies of interaction residing at the coresponding intersubunit boundaries. These

[2] Oxygenation-sensitive constraints are defined as those constraints that are altered by the state of oxygenation of hemoglobin. Examples of these are the $\alpha^i\beta^i$, $\alpha^i\beta^j$, $\alpha\alpha$, and $\beta\beta$ constraints of the order of free energy couplings concepts that are altered depending on the state of ligation of the individual α and β subunits.

Table I: Constraints to Binding with Increasing Ligation for First- and Second-Order Free Energy Couplings

subunits liganded	remaining constraints to binding		
	first-order coupling[a]	second-order coupling[a]	g_{ij}
none	2 $\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$	2 $\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$	1
α	$\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\beta\beta\}$	2 $\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$	2
β	$\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$	2 $\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$	2
$\alpha\alpha$	$\{\beta\beta\}$	2 $\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\beta\beta\}$	1
$\beta\beta$	$\{\alpha\alpha\}$	2 $\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$	1
$\alpha^i\beta^i$	$\{\alpha^i\beta^i\}$	$\{\alpha^i\beta^i\}$, 2 $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$	2
$\alpha^i\beta^j$	$\{\alpha^i\beta^j\}$	2 $\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$	2
$\alpha\alpha\beta$	0	$\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\beta\beta\}$	2
$\alpha\beta\beta$	0	$\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$	2
$\alpha\alpha\beta\beta$	0	0	1

[a] The 2's in the columns below refer to the number of constraints of the particular type.

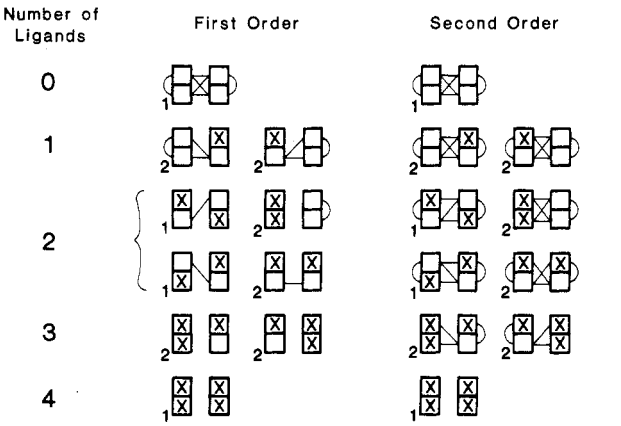


FIGURE 2: Alterations in intersubunit interactions, as in Table I, for each of the 10 possible partially and fully oxygenated tetrameric states. An oxygenated subunit is depicted with an X. The numbers at the lower left of each of the species denote the statistical degeneracy of that species.

are shown diagrammatically in Figure 1. These free energies of interaction, or thermodynamic constraints, are defined to exist if neither of the paired subunits is oxygenated. For first-order free energy coupling, these constraints are released if either of the paired subunits is oxygenated. For second-order free energy coupling, the constraints are released when both of the subunits are oxygenated. Therefore, for these types of interactions the reference state is defined to be the fully oxygenated tetramer. In this work, as in the previous work (Weber, 1984), it is assumed that $\{\alpha^1\beta^1\} = \{\alpha^2\beta^2\}$ and $\{\alpha^1\beta^2\} = \{\alpha^2\beta^1\}$. The free energies of these two types of interactions are referred to as $\{\alpha^i\beta^i\}$ and $\{\alpha^i\beta^j\}$, respectively, where i is not equal to j. Table I is a summary of the number of constraints of each of these types for both first- and second-order free energy couplings for each of the 10 possible ways to partially or fully oxygenate the hemoglobin tetramer. Figure 2 shows these interactions for first- and second-order couplings at each state of ligation.

Two additional parameters are required to fully describe the binding of oxygen to tetrameric hemoglobin. These two parameters, $\delta_{4\alpha}$ and $\delta_{4\beta}$, are the free energies of interaction of oxygen with the individual α and β subunits in tetrameric hemoglobin. These composite parameters must include all interactions within the hemoglobin tetramer that are not specifically addressed by the $\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, and $\{\beta\beta\}$ described above. More specifically, the $\delta_{4\alpha}$ and $\delta_{4\beta}$ parameters are composites of a large number of interactions, a few of which are the following: (1) One interaction is the local chemical affinity of the heme for oxygen. (2) Another type of interaction includes all constraints, both oxygenation-sen-

sitive and -insensitive, imposed on the heme by the α- and β-globins. This would include, for example, the tertiary structural changes of the individual subunits that occur upon oxygenation. Another example of this general type of constraint is the energy required for the alteration of the salt bridge that is internal to the β subunit but does not exist in the α subunit (Baldwin & Chothia, 1979; Perutz et al., 1969). (3) Another type of interaction includes any oxygenation-insensitive constraints imposed along the $\alpha^1\beta^1$ interface when the α and β subunits associate to form the $\alpha\beta$ dimers.

Since these two parameters, $\delta_{4\alpha}$ and $\delta_{4\beta}$, are actually composites of a large number of different types of interactions, it is unrealistic to assume that both parameters will necessarily have the same numerical value. Several recent publications (Peller, 1982; Weber, 1982, 1983, 1984) have attempted to link the molecular origin of the asymmetry[3] of ligand binding data to functional differences in the α and β subunits. It has also been noted (Nobel, 1983) that the allosteric model of Monod, Wyman, and Changeux (Monod et al., 1965) predicts asymmetrical binding isotherms without asymmetry of the subunits. In light of the possibility of functional differences in the subunits it is important that $\delta_{4\alpha}$ and $\delta_{4\beta}$ *not be required* to have the same meaning or numerical value.

In addition, the numerical values of these parameters, $\delta_{4\alpha}$ and $\delta_{4\beta}$, are dependent on the choice of the reference state. For example, assume that the deoxygenated tetramer is taken as a reference state and that it has no intramolecular interactions. The free energy couplings concept would then predict a series of interactions analogous to $\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, and $\{\beta\beta\}$ that are formed, rather than broken, upon oxygenation. The numerical values of these free energies of interaction would be of the opposite sign while $\delta_{4\alpha}$ and $\delta_{4\beta}$ would have significantly different values.

The free energy couplings concept presented here requires six parameters to characterize the binding of oxygen to the hemoglobin tetramer: $\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$, $\delta_{4\alpha}$, and $\delta_{4\beta}$. Since the tetramer has only four oxygen binding sites, its binding properties can also be completely defined, for a single set of experimental conditions, by four stepwise Adair binding constants:[4] k_{41}, k_{42}, k_{43}, and k_{44}. Consequently, the translation of the coupling free energies into Adair constants involves four equations, one to define each of the Adair constants, with each equation involving six variable free energies: $\{\alpha^i\beta^i\}$, $\{\alpha^i\beta^j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$, $\delta_{4\alpha}$, and $\delta_{4\beta}$. This implies that there is a large number of sets of free energies of the thermodynamic constraints that will map into the same four Adair constants and thus correctly describe the actual oxygen binding data at a single set of experimental conditions. Therefore, some additional assumptions must be included to generate a statistically valid least-squares fit of the actual experimental data to these concepts (see Results).

Two additional parameters, $\delta_{2\alpha}$ and $\delta_{2\beta}$, pertain to the properties of the dimeric oligomers and are analogous to the tetramer parameters, $\delta_{4\alpha}$ and $\delta_{4\beta}$. These dimer parameters are composites of a number of the same interactions that are summed into the corresponding tetramer parameters. It cannot be assumed a priori that $\delta_{2\alpha}$ and $\delta_{2\beta}$ have the same conceptual meaning as the respective tetramer parameters or that they have the same numerical values. Interactions between the α and β subunits could also occur in the dimeric oligomers. It should be noted that the interactions between α and β subunits of the dimers need not be identical with the $\alpha^i\beta^i$ interaction within the tetrameric oligomer. The free energy couplings concept thus requires three parameters to describe the oxygen binding properties of the dimeric oligomers. As with the tetramers, this is more parameters than can be accommodated by the thermodynamic formulation. However, previous work [cf. Mills & Ackers (1979)] has shown that the dimeric oligomers bind oxygen noncooperatively. We have therefore assumed, as Weber did in his report on the order of free energy couplings (Weber, 1984), that $\delta_{2\alpha}$ is equal to $\delta_{2\beta}$ and that no oxygenation-sensitive interactions exist in dimeric oligomers.

The free energy couplings concept does not explicitly include a formulation of the interactions involved in the dimer to tetramer polymerization that are not sensitive to oxygenation. Consequently, a mathematical formulation of the concept requires that one of the dimer to tetramer free energies of association be included. We chose ${}^0\Delta G_2$, the free energy to form deoxygenated tetramers from deoxygenated dimers, because it has been measured independently under the same experimental conditions (Ip et al., 1976) as the Mills et al. data.

Mathematical Formulation

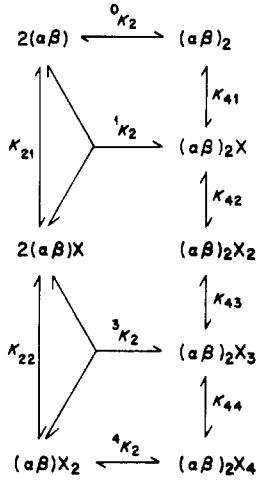
The translation of the thermodynamic concept of order of free energy couplings into a mathematical formula that describes (1) fractional saturation as a function of oxygen and hemoglobin concentration and/or (2) free energy of association as a function of oxygen concentration requires two types of information. First, the allowed types of thermodynamic interactions between individual constituents of the molecule must be explicitly defined. These free energies of interaction are sometimes referred to as thermodynamic constraints upon the system. Second, there must be an explicit statement of the rules by which these constraints are altered during the functional cycle. For the present example the functional cycle is the oxygenation–deoxygenation of the hemoglobin and its concomitant subunit dissociation and association. Once the constraints and rules have been defined, an equation that describes the fractional saturation with oxygen as a function of oxygen concentration and protein concentration can be formulated.

Least-squares techniques are then used to evaluate the numerical values of the parameters of the free energy couplings concept that best describe the experimental data. The least-squares procedure that was used for the estimation of these parameters from the raw experimental data has been presented elsewhere (Atha et al., 1979; Johnson, 1983; Johnson & Ackers, 1977, 1982; Johnson et al., 1976, 1981; Johnson

[3] Asymmetry of ligand binding describes the shape of the ligand binding isotherm when plotted as bound vs. log ligand concentration. It does not refer to the structural orientation of the ligands as they are bound to the hemoglobin tetramer. For a rigorous mathematical definition the reader is referred to Weber (1982).

[4] The stepwise Adair binding constants of tetrameric hemoglobin, k_{4i}, are defined as the association constant of the ith oxygen to a tetramer with $i-1$ oxygens already bound. These are sometimes expressed as free energies, ΔG_{4i}. Analogous terms are used for the dimeric oligomers, k_{2i} and ΔG_{2i}. It should also be noted that the intrinsic free energy changes, $\Delta G_{4i}'$, are sometimes utilized. The difference between $\Delta G_{4i}'$ and ΔG_{4i} is that the macroscopic constant, ΔG_{4i}, describes the binding of oxygen to tetrameric hemoglobin and the intrinsic constant, $\Delta G_{4i}'$, describes the average affinity of oxygen to an individual chain within the tetramer. The difference is a redundancy factor that describes the number of ways that a hemoglobin with $i-1$ oxygens already bound can bind an additional oxygen. For example, since unliganded tetramers can bind oxygen to four different sites, a redundancy factor of $RT \ln 4$ is subtracted from the macroscopic constant, ΔG_{41}, in order to calculate the intrinsic constant, $\Delta G_{41}'$. For a more complete definition of this particular formulation of these parameters the reader is referred to the literature (Ackers, 1980; Ackers & Halvorson, 1974; Ackers & Johnson, 1981; Atha et al., 1979; Chu & Ackers, 1981; Chu et al., 1984; Flanagan et al., 1981; Ip et al., 1976; Johnson & Ackers, 1977, 1982; Johnson et al., 1976, 1984; Mills & Ackers, 1979; Mills et al., 1976; Pettigrew et al., 1982; Smith & Ackers, 1983; Valdes & Ackers, 1978).

Scheme I



& Frasier, 1985) and will not be restated here.

Once the constraints and rules have been defined, as in the previous section, there are two alternative formalisms that can be employed to translate the thermodynamic concepts into an equation describing the oxygen binding data. One approach uses statistical thermodynamic partition functions (Herzfeld & Stanley, 1974; Hill, 1960; Johnson & Ackers, 1977; Johnson et al., 1984; Lee & Karplus, 1983; Nobel, 1983; Peller, 1982; Szabo & Karplus, 1972). The other approach is the procedure outlined by Weber, based on the differences in the mean Gibbs energies of hemoglobin species in various oxygenated states (Weber, 1972, 1975, 1982, 1983, 1984), and will be referred to as the mean Gibbs energy method. These two methods yield quantitatively different results that are, in some cases, qualitatively similar. Consequently, an analysis of both the first- and second-order free energy couplings for both methods of formulation is presented under Results and Discussion.

Both methods of formulating the binding isotherm consist of a two-step process. In both methods the first step is to translate the mechanistic parameters of the first- and second-order free energy couplings concepts into a set of seven independent thermodynamic constants of the dimer–tetramer linkage as depicted in Scheme I. The second step is to utilize the previously published binding isotherm for these thermodynamic constants (Ackers & Halvorson, 1974).

(A) Linkage Scheme. The ligand-linked subunit assembly of human hemoglobin A can be described in terms of model-independent Adair constants (Ackers & Halvorson, 1974) as shown in Scheme I, where the ${}^iK_2$ 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; Johnson & Ackers, 1977; Johnson et al., 1976; Mills & Ackers, 1979; Mills et al., 1976; Valdes et al., 1978). Here I present only a brief statement of the relationships to be used in the analyses.

The mathematical form of the binding isotherm for the ligand-linked dimer–tetramer association system of Scheme I is (Ackers & Halvorson, 1974)

$$\bar{Y}_{2,4} = \frac{Z_2' + Z_4'(\sqrt{Z_2^2 + 4{}^0K_2Z_4[P_t]} - Z_2)/(4Z_4)}{Z_2 + \sqrt{Z_2^2 + 4{}^0K_2Z_4[P_t]}} \quad (1)$$

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

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

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

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

$[P_t]$ 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_{2i} and K_{4i} are the product Adair constants for dimers and tetramers, respectively. Formulation of any mechanism for the hemoglobin system depicted in Scheme I consists of defining the relationships between the parameters of the particular mechanism and the model-independent phenomenological Adair contants of eq 1–5 (Ackers & Johnson, 1981).

It should be noted that eq 2 and 4 are the same as the binding polynomials formulated by Wyman (1948, 1964). In this case eq 2 represents a species that can bind two ligands, and eq 4 is for a species that can bind four ligands. It should also be noted that Wyman's binding polynomials and our eq 2 and 4 are the macroscopic analogue of the particular grand canonical partition functions.

(B) Statistical Thermodynamic Formulation. When oxygen is used as a ligand, the partition function of hemoglobin tetramers is (Johnson & Ackers, 1982)

$$\Xi_4 = \sum_i \sum_j g_{ij} e^{-G_{ij}/(RT)} [X]^i \quad (6)$$

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. R is the gas constant and T is the absolute temperature. The values of the statistical degeneracies of each of the distinguishable macroscopic states of ligation are given in Table I and Figure 2. In eq 6, [X] is the concentration of the unbound oxygen. The relative probability of each of the species is given in terms of a Boltzmann distribution of the free energy of that species. The quantity ξ_{ni} is a macroscopic analogue of the grand canonical partition function [cf. Hill (1960)] for the subsystem comprised of n-mers with i ligands bound and is defined by

$$\xi_{ni} = \sum_j g_{ij} e^{-G_{ij}/(RT)} \quad (7)$$

The relationship between the subsystem partition function ξ_{ni} and the other system properties is readily seen by noting that the expression on the right of eq 6 may be written as a sum of terms in increasing powers of [X], from 0 to 4.

$$\Xi_4 = \xi_{40} + \xi_{41}[X] + \xi_{42}[X]^2 + \xi_{43}[X]^3 + \xi_{44}[X]^4 \quad (8)$$

A term by term comparison of this expression with eq 4, which is a macroscopic analogue of the grand partition function in terms of product Adair constants, yields

$$K_{4i} = \xi_{4i}/\xi_{40} \quad (9)$$

Analogous relationships are used for dimers.

Substituting eq 9 into eq 8 yields the partition function in terms of the product Adair constants:

$$\Xi_4 = 1 + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4 \quad (10)$$

The mean number of ligands bound, $\bar{N}_4$, can be expressed in terms of the partition function as (Hill, 1960)

$$\bar{N}_4 = \partial \ln \Xi_4 / \partial \ln [X] \quad (11)$$

Applying the transformation, eq 11, to eq 10 yields the well-known Adair equation:

$$\bar{N}_4 = \sum_{i=1}^{4} iK_{4i}[X]^i / (1 + \sum_{i=1}^{4} K_{4i}[X]^i) = Z_4'/Z_4 \quad (12)$$

Table II: Intrinsic ΔG_{4i} Values from Literature References

	Mills et al. (1976)	Chu et al. (1984)
${}^0\Delta G_2$	−14.38 ± 0.2[a]	−14.35 ± 0.1
$\Delta G_{21}'$	−8.38 ± 0.28	−8.35 ± 0.1
$\Delta G_{22}'$	−8.38 ± 0.21	−8.35 ± 0.1
$\Delta G_{41}'$	−5.45 ± 0.21	−5.43 ± 0.11
$\Delta G_{42}'$	−5.28 ± 0.54	−5.54 ± 1.3
$\Delta G_{43}'$	−7.80 ± 0.63	−6.96 ± 1.1
$\Delta G_{44}'$	−8.65 ± 0.42	−9.16 ± 0.35
σ^2	2.25×10^{-5}	1.70×10^{-5}

[a] Corresponds to a 1 standard deviation confidence interval.

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 are evaluated as the sum of all of the thermodynamic interactions within a given distinguishable species relative to the reference state.

The resulting values of K_{4i} and K_{2i} and the value of ${}^0K_2$ derived from the independently measured ${}^0\Delta G_2$ are then utilized with eq 1–5 to evaluate the fractional saturation of hemoglobin as a function of oxygen and hemoglobin concentration.

(C) Mean Gibbs Energy Formulation. The first step in this formulation, which is the method used by Weber (1984), is to calculate a mean Gibbs energy $\overline{\Delta G_i}$ at each state of ligation. For tetramers, these are defined as

$$\overline{\Delta G_i} = \sum_j G_{ij} g_{ij} e^{-G_{ij}/(RT)} / \xi_{4i} \qquad (13)$$

where G_{ij}, g_{ij}, and ξ_{4i} are as defined in the statistical thermodynamic formulation. The macroscopic Adair constants for the binding of oxygen to tetramers are then evaluated as

$$K_{4i} = e^{-(\overline{\Delta G_i} - \overline{\Delta G_0})/(RT)} \qquad (14)$$

Analogous relationships are used for dimers. As with the statistical thermodynamic formulations, the values of K_{4i} and K_{2i} are used in conjunction with ${}^0K_2$ to evaluate the fractional saturation of oxygen.

(D) Comparison of the Formulations. The statistical thermodynamic formulation and the mean Gibbs energy formulation are identical if, and only if, the hemoglobin tetramer can exist in only five possible configurations: unliganded, single liganded, double liganded, triple liganded, and fully liganded. If it is possible for multiple distinguishable configurations of the hemoglobin tetramer with a particular degree of ligation to exist, then the two formulations average these multiple configurations differently. The formulation of the concepts of first- and second-order free energy couplings involves two distinguishable singly liganded species, four distinguishable doubly liganded species, and two distinguishable triply liganded species (see Table I and Figure 2). As a consequence, the mathematical descriptions of the binding isotherm are different for the two formulations.

Results and Discussion

Table II presents the intrinsic free energy changes that define the binding of oxygen to stripped hemoglobin A as derived from the two sets of previously published oxygen binding and kinetic observations (Chu et al., 1984; Mills et al., 1976). Weber used the free energy changes reported by Mills et al. (1976) in his analysis of the order of free energy couplings in human hemoglobin (Weber, 1984). In the present report the original oxygen binding data as a function of hemoglobin and oxygen concentration is used in conjunction with ${}^0K_2$. Using the actual data makes it possible to include the experimental uncertainties of the data into the analysis of the order of free energy couplings. The confidence intervals of the derived free energy changes, as presented in Table II, embody a complex cross-correlated average of the experimental uncertainties of the actual data and as such are extremely difficult to treat correctly in an analysis that fits parameters to these equilibrium constants rather than actual data.

As previously noted, the mathematical description of the first- and second-order free energy couplings concepts consists of ten adjustable parameters: six parameters that describe the binding of oxygen to the tetrameric oligomer of hemoglobin, three parameters that describe the binding of oxygen to the dimeric oligomer, and a constant to describe the dimer–tetramer association. The previous model-independent thermodynamic analysis (Ackers & Halvorson, 1974) indicates that the linkage between oxygen binding and polymerization can be completely described by the seven free energy changes presented in Table II. Thus, of the ten parameters needed to formulate the free energy couplings concepts only seven are independent. As noted in the description of the model, two of these parameters can be eliminated by the assumption that the dimers bind oxygen noncooperatively: $\Delta G_{21} = \Delta G_{22}$. However, an assumption is still required to map uniquely the six parameters describing the order of free energy couplings for the binding of oxygen to tetrameric hemoglobin into the four Adair constants. When reasonable values for two of the parameters are assumed, the remaining four parameters can be estimated by the least-squares procedure.

The X-ray crystallographic structures (Baldwin & Chothia, 1979) of liganded and unliganded hemoglobin tetramers yield information that can be used for assumptions about two of the six parameters. First, in both the liganded and unliganded structures the interactions between the α and β subunits of the $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers appear to be identical. As a consequence, I have assumed, as Weber (1984) did, that the parameter $\{\alpha^i\beta^i\}$ has a value of 0. Second, in both the liganded and unliganded structures the two β subunits are not in physical contact with each other. It is therefore reasonable to assume that the interaction free energy between the two β subunits, the $\{\beta\beta\}$ free energy change, is 0. The least-squares parameter estimation procedure is then used to evaluate the remaining parameters that will best describe the experimental data, subject to the stated assumptions. To demonstrate that second-order free energy coupling is consistent with the actual experimental data, all that is required is that one set of parameters be found that allows the second-order free energy coupling to fit the raw experimental data to within the same precision as the first-order free energy coupling and the original thermodynamic analysis in terms of Adair constants.

Table III presents an analysis of the Mills et al. (1976) data by both formulations of the first- and second-order free energy couplings concepts. For these analyses the values of ${}^0\Delta G_2$, $\delta_{2\alpha}$, and $\delta_{2\beta}$ were taken to be the values that gave the lowest variance from the thermodynamic analysis in terms of Adair constants (see Table II). It is apparent that all four of these calculations yield the same variance and cannot be distinguished on that basis. It should also be noted that the free energies corresponding to the Adair constants, ΔG_{4i}, that are calculated from these analyses are in agreement with each other and with the model-independent thermodynamic parameters.

To demonstrate that the above conclusions are not dependent on a specific set of data, these four analyses were also performed on the set of data presented by Chu et al. (1984). As above, all four calculations (Table IV) resulted in as good

Table III: Free Energies (kcal/mol) of the First- and Second-Order Constraints from Analysis of the Mills et al. (1976) Data

	mean Gibbs energy		statistical thermodynamic	
	first order	second order	first order	second order
		Fitted Parameters		
$\delta_{4\alpha}$	−8.37 (−8.22, −8.50)[b]	−4.07 (−3.15, −4.55)	−9.36 (−9.27, −9.45)	−2.98 (−2.74, −3.19)
$\delta_{4\beta}$	−8.37[c]	−6.33 (−6.30, −6.36)	−9.36[c]	−5.87 (−5.84, −5.91)
$\{\alpha\alpha\}$	−2.01 (−1.64, −2.36)	3.22 (2.29, 4.86)	−3.01 (−2.63, −3.36)	4.13 (3.40, 4.99)
$\{\beta\beta\}$[d]	0.00	0.00	0.00	0.00
$\{\alpha^i\beta^{ii}\}$[d]	0.00	0.00	0.00	0.00
$\{\alpha^i\beta^i\}$	−2.08 (−1.97, −2.17)	1.64 (1.59, 1.74)	−3.57 (−3.49, −3.64)	2.73 (2.50, 2.97)
σ^2	2.46×10^{-5}	2.46×10^{-5}	2.48×10^{-5}	2.46×10^{-5}
		Derived Parameters[a]		
$\Delta G_{41}'$	−5.42	−5.47	−5.39	−5.47
$\Delta G_{42}'$	−6.10	−5.78	−6.22	−5.78
$\Delta G_{43}'$	−6.61	−7.11	−6.33	−7.11
$\Delta G_{44}'$	−9.18	−8.94	−9.36	−8.94

[a] $^0\Delta G_2$, ΔG_{21}, and ΔG_{22} were assumed to be the thermodynamic values presented in Table II. [b] The numbers in parentheses correspond to a 1 standard deviation confidence interval. These are present only for the parameters that were actual fitting parameters. [c] Assumed to have the same value as $\delta_{4\alpha}$. [d] Assumed values; see text for reasons for the assumptions.

Table IV: Free Energies (kcal/mol) of the First- and Second-Order Constraints from Analysis of the Chu et al. (1984) Data

	mean Gibbs energy		statistical thermodynamic	
	first order	second order	first order	second order
		Fitted Parameters		
$\delta_{4\alpha}$	−9.07 (−8.70, −9.69)[c]	−3.41[e]	−9.74 (−9.34, −10.11)	−1.58 (−0.16, −2.33)
$\delta_{4\beta}$	−9.07[d]	−6.28 (−6.27, −6.29)	−9.74[d]	−5.87[e]
$\{\alpha\alpha\}$	−3.75 (−3.02, −4.97)	3.14 (2.62, 3.68)	−4.33 (−3.44, −5.17)	3.87 (3.60, 4.06)
$\{\beta\beta\}$[b]	0.00	0.00	0.00	0.00
$\{\alpha^i\beta^{ii}\}$[b]	0.00	0.00	0.00	0.00
$\{\alpha^i\beta^i\}$	−2.78 (−2.41, −3.40)	2.21 (1.95, 2.48)	−3.83 (−3.48, −4.15)	4.09 (3.26, 5.65)
σ^2	1.28×10^{-5}	1.33×10^{-5}	1.24×10^{-5}	1.28×10^{-5}
		Derived Parameters[a]		
$\Delta G_{41}'$	−5.47	−5.45	−5.51	−5.47
$\Delta G_{42}'$	−5.78	−5.77	−5.63	−5.75
$\Delta G_{43}'$	−5.83	−6.32	−6.10	−6.03
$\Delta G_{44}'$	−9.88	−9.41	−9.74	−9.71

[a] $^0\Delta G_2$, ΔG_{21}, and ΔG_{22} were assumed to be the thermodynamic values presented in Table II. [b] Assumed values; see text for reasons for the assumptions. [c] The numbers in parentheses correspond to a 1 standard deviation confidence interval. These are presented only for the parameters that were actual fitting parameters. [d] Assumed to have the same value as $\delta_{4\alpha}$. [e] The parameters from this fit were so highly cross-correlated that it was impossible to evaluate a reasonable confidence interval.

a variance as the original thermodynamic analysis (Table II), and predicted, within the experimental uncertainties, the same values of the ΔG_{4i}'s as the thermodynamic analysis. The results of these analyses are shown in Table IV.

Based solely on the Mills et al. and Chu et al. data sets, the order of free energy coupling could be either first or second order since there is no basis to assign a priori values to the parameters. However, on the basis of information other than the actual Mills et al. and Chu et al. data, it might possibly be argued that the values of the composite parameters $\delta_{4\alpha}$ and $\delta_{4\beta}$ are unrealistically small for second-order free energy coupling. If additional information is to be employed to distinguish first-order from second-order free energy couplings, then the entire body of experimental information pertaining to the oxygenation of human hemoglobin must be considered. There are at least three experimental observations that are difficult, if not impossible, to reconcile with either the first- or the second-order free energy coupling concept.

First, the model of Monod, Wyman, and Changeux (Monod et al., 1965) is inconsistent with the order of free energy couplings concept. However, from the X-ray crystallographic structures (Baldwin & Chothia, 1979; Perutz et al., 1969) it is known that hemoglobin undergoes at least a two-state transition similar to what is described by the Monod, Wyman, and Changeux model (Monod et al., 1965). This inconsistency could be interpreted as indicating that neither first- nor second-order free energy coupling is capable of describing the multistate structural transition that is known to exist in hemoglobin (Noble, 1983).

If it is assumed that the values of $\delta_{2\alpha}$, $\delta_{2\beta}$, $\delta_{4\alpha}$, and $\delta_{4\beta}$ are all equal, then another inconsistency is introduced. It is an experimental observation that the last oxygen binds to the tetrameric oligomer with a higher affinity than the oxygens that bind to the dimeric oligomer. This phenomenon is called "quaternary enhancement" (Chu et al., 1984; Johnson et al., 1984; Mills & Ackers, 1979; Valdes & Ackers, 1978). This phenomenon is evident in the model-independent thermodynamic parameters presented in Table II. The only way to accommodate this phenomenon into the order of free energy couplings concept is to assume that $\delta_{4\alpha}$ is not equal to $\delta_{2\alpha}$ and/or that $\delta_{4\beta}$ is not equal to $\delta_{2\beta}$.

A recent paper has experimentally resolved the cooperative free energy of oxygen binding at each of the 10 ligation states of human hemoglobin (Smith & Ackers, 1985). This work has shown that the hemoglobin tetramer functions as a "combinatorial switch" when it is "ligated" by conversion into the cyanomet form ($Fe^{3+}CN$). The functional cycle of this combinatorial switch is inconsistent with both first- and second-order free energy couplings. It should be noted that CN and O_2 are not the same ligand so this inconsistency is only indicative rather than a disproof of the concepts of first- and second-order free energy couplings.

In conclusion, it has been shown that the second-order free energy coupling concept (Weber, 1984) is consistent with the

original oxygen binding data. Therefore, the assertion that the first-order free energy coupling concept is the only possible mechanistic description of the functional cycle of human hemoglobin (Weber, 1984) is not justified.

Registry No. Hemoglobin A, 9034-51-9; O_2, 7782-44-7.

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