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The Linkage Between Oxygenation and Subunit Dissociation in Human Hemoglobin

(protein association/ligand binding/thermodynamics)

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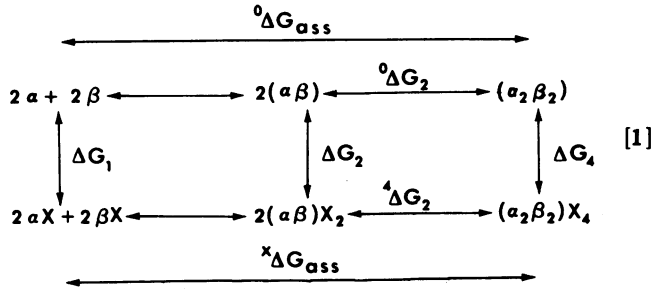
ABSTRACT **The use of subunit dissociation as a means of probing intersubunit contact energy changes which accompany cooperative ligand binding has been studied for the case of human hemoglobin. An analysis is presented delineating the information that can be obtained from the linkage relationships between ligand binding and subunit dissociation of hemoglobin tetramers into dimers. The analysis defines (a) the variation of the saturation function, $\bar{Y}$, with total protein concentration, (b) the variation of the subunit dissociation constant ${}^{z}K_2$ with ligand concentration (X) and (c) the correlations between changes in dimer–dimer contact energy and the sequential ligand binding steps. Sensitivity of the linkage functions has been explored by numerical simulation. It is shown that subunit dissociation may appreciably affect oxygenation curves under usual conditions of measurement and that relying solely on either ${}^{z}K_2$ or $\bar{Y}$ may lead to incorrect pictures of the energetics, whereas the combination defines the system much more exactly.**

The correlation of energy changes and quaternary structure changes with the sequence of oxygen binding steps is a central problem in hemoglobin research. The major transformations in quaternary structure upon oxygenation are known to occur via changes in the intersubunit contact region separating ($\alpha^1\beta^1$) and ($\alpha^2\beta^2$) dimer pairs (1, 2). It is here that stabilizing interactions, present in the "constrained" deoxy quaternary structure, are abolished upon oxygenation. A determination of the amount of energy expended within this contact region at each stage of oxygenation is of crucial importance to understanding the mechanism of cooperativity. There is now considerable evidence that the dissociation of tetramer at neutral pH occurs via cleavage along the same contact region to form ($\alpha\beta$) dimer pairs (3–8). Even at the lowest concentrations, dissociation into individual α and β chains does not occur to appreciable degree unless the tertiary structures are disrupted (6, 9). Moreover, since the tetramer-dimer dissociation constant varies with oxygenation over approximately six orders of magnitude (10–12), its measurement as a function of oxygenation would appear to offer an extremely sensitive approach to the resolution of energy changes within the contact region. These considerations and the recent improvement in techniques capable of studying subunit dissociation (13) and oxygenation (14, 15) in extremely dilute solution, have made necessary a study of the information that may be obtained from experimental measurements. In this paper we present such an analysis of the linkage between oxygen binding and tetramer-dimer subunit dissociation.

The linkage principles which underlie the present analysis have been developed extensively by Wyman (16) and certain applications to hemoglobin have been previously made (10–12, 17, 18). However, no analysis has been formulated for resolving the experimentally determinable linkage functions into cooperative energy terms for the sequential ligand binding steps. The experimental properties to be considered are: (a) the variation of the saturation function Y with total protein concentration (P_t), and (b) the variation of the subunit dissociation constant ${}^{z}K_2$ with ligand concentration (X). These functions are then used to obtain correlations between changes in dimer–dimer contact energy and the sequential binding steps. The theory is model-independent, but serves to produce constraints which must be obeyed by any model purporting to explain the behavior of hemoglobin.

THE OVERALL ENERGY BALANCE

The overall energy balance for ligand binding and subunit association is shown below.



[1]

In this scheme, ΔG_4 is the total free energy of binding four ligand molecules X onto the tetramer, and ΔG_1 is the corresponding energy for complete ligation of dissociated α and β chains. The difference between ΔG_4 and ΔG_1 is equal to the difference between energies of association for liganded and unliganded tetramers obtained from the corresponding monomeric species.

$$\Delta G_4 - \Delta G_1 = {}^{z}\Delta G_{\text{assoc.}} - {}^{0}\Delta G_{\text{assoc.}}$$

Several recent studies have either adopted (11, 12) or tried to evaluate (8, 19, 20) the assumption that combining α and β chains into ($\alpha\beta$) dimers does not change their oxygen affinities, so that ΔG_2 is quantitatively the same as ΔG_1. Based on the assumption that $\Delta G_2 = \Delta G_1$, Thomas and Edelstein (11, 12) estimated the deoxy constant ${}^{0}K_2$ from experimentally known ΔG_4, ${}^{4}G_2$, and ΔG_1. [The association constant ${}^{4}K_2$ for liganded hemoglobin A had been determined directly to be about 5×10^5 M^{-1} in low salt ($\leq$0.2 M), neutral pH, and 20°; ${}^{4}\Delta G_2 = -7.5$ kcal/mole of heme (3–7).] The resulting value of 3×10^{11} M^{-1} for ${}^{0}K_2$ (${}^{0}\Delta G_2 = -15.5$ kcal/mole of heme) was found to be consistent with data on the variation of carbon monoxide binding at low protein concentration (11, 12).

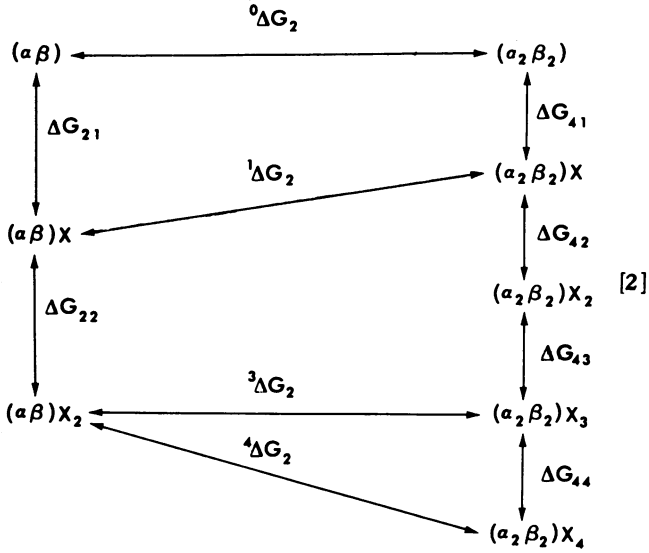
Although the assumption that $\Delta G_2 = \Delta G_1$ may be valid under certain conditions, there is presently no reason to believe in its general validity. It should be noted that the binding energies ΔG_1, ΔG_2, and ΔG_4 refer to the respective median ligand activities (16). The assumption that $\Delta G_2 = \Delta G_1$ is not the same as assuming that dimer binds ligand noncooperatively. The latter means that the two successive binding constants of dimer differ only by a statistical factor. In the conceptual analysis to be presented in this paper, *we do not assume either that $\Delta G_2 = \Delta G_1$ or that any particular relation exists between successive binding affinities.*

THEORY

The quantity ${}^4\Delta G_2 - {}^0\Delta G_2$ which arises from the right side of scheme [1] represents the energy change within the dimer-dimer contact region upon oxygenation of the tetramer. To develop a thermodynamic mechanism for the expenditure of cooperative energy in the hemoglobin molecule, it is necessary to resolve this energy difference (${}^4\Delta G_2 - {}^0\Delta G_2$) into the cooperative energy changes accompanying each successive binding step. This resolution involves two stages: (*A*) specifying the linkage relationships and the experimentally derivable equilibrium constants for the system, and (*B*) analyzing these equilibrium constants in terms of the desired structural and energetic correlations.

A. Linkage between oxygenation and subunit dissociation

The equilibrium states of hemoglobin dimers and tetramers are shown below and the energetic relationships between them are diagrammed.



[2]

In this scheme the various liganded states of tetramer are shown on the *right* and those of dimer on the *left*. A state designated $(\alpha_2\beta_2)X_i$ (i = 1,2,3,4), represents all tetrameric species having i ligands are distributed differently. The species designations of scheme [2] and the corresponding energy relationships are well-defined averages over many possible microscopic states.

To resolve the equilibria in this scheme, at least seven equilibrium constants must be known. The eight equilibria on the outer sides of the scheme are related directly to the experimentally measureable subunit dissociation constant ${}^xK_2$ and to the measureable binding isotherm, $\bar{Y}$, through the linkage relationships described below.

1. The Saturation Function. The fraction $\bar{Y}$ of sites with bound oxygen, as a function of free oxygen concentration (X) and molar concentration of heme (P_T) may be expressed:

$$\bar{Y} = \frac{Z'_2 + Z'_4\,(\sqrt{Z_2^2 + 4^0K_2Z_4(P_T)} - Z_2)/4Z_4}{Z_2 + \sqrt{Z_2^2 + 4^0K_2Z_4(P_T)}} \qquad [3]$$

where

$$Z_2 = 1 + K_{21}(X) + K_{22}(X)^2$$
$$Z'_2 = K_{21}(X) + 2K_{22}(X)^2$$
$$Z_4 = 1 + K_{41}(X) + K_{42}(X)^2 + K_{43}(X)^3 + K_{44}(X)^4$$
$$Z'_4 = K_{41}(X) + 2K_{42}(X)^2 + 3K_{43}(X)^3 + 4K_{44}(X)^4.$$

It can be seen that $\bar{Y}$ is a function of the seven constants (${}^0K_2$, K_{21}, K_{22}, K_{41}, K_{42}, K_{43}, K_{44}) which correspond to the free energies shown in scheme [2]. The K_{4i} are Adair constants for oxygen binding to tetramer and the K_{2i} are Adair constants for dissociated dimer species (see *Appendix* for exact definitions).

2. The Subunit Association Constant. The overall macroscopic dimerization constant for subunit association is

$${}^xK_2 = \frac{[\text{total tetrameric species}]}{[\text{total dimeric species}]^2} = {}^0K_2\,\frac{Z_4}{(Z_2)^2}. \qquad [4]$$

This quantity is comprised of individual constants for the various equilibria, including the oxygen binding equilibria.

As a function of oxygen concentration, ${}^xK_2$ ranges from the deoxy subunit association constant, ${}^0K_2$, down to the oxy constant, represented by the limit:

$${}^4K_2 = \lim_{(x)\to\infty} {}^xK_2 = {}^0K_2\,\frac{K_{44}}{(K_{22})^2}. \qquad [5]$$

Equation [5] states conservation of energy around the outer sides of scheme [2]. The large variation of ${}^xK_2$ with (X), i.e., between 10^5 and 10^{11}, makes this an extremely sensitive property. Combined with other data it can be used to resolve the seven constituent equilibrium constants.

A second useful property of this function is given by the linkage relationship:

$$\frac{d\ln {}^xK_2}{d\ln(X)} = 4\,(\bar{Y}_4 - \bar{Y}_2). \qquad [6]$$

It should be noted that $\bar{Y}_2$ can be constructed in a completely empirical fashion from experimental knowledge of ${}^xK_2$ and $\bar{Y}_4$ and does not require evaluating the constituent terms.

In principle, a precise determination of either $\bar{Y}$ versus (P_T) or ${}^xK_2$ versus (X) could yield the seven constants necessary to define scheme [2]. A much more promising strategy would be to utilize all of the functions represented by Eqs. [3–6]. An exploration of this strategy will be presented elsewhere.

B. Correlation of energy terms with binding steps

If one knows the eight free energy terms on the outer sides of scheme [2], the next stage of analysis is to correlate appropriate energy terms with the sequential oxygen binding steps. To do this the scheme must be divided into three regions representing the first binding step, the second and third binding steps together, and the fourth binding step.

1. Binding the First Oxygen. The subunit association energy for formation of singly-liganded tetramer is ${}^1\Delta G_2 = \Delta G_{41} - \Delta G_{21} + {}^0\Delta G_2$. This energy is uniquely defined since there is only one way to form a singly liganded tetramer: $(\alpha\beta)X + (\alpha\beta) \leftrightarrows (\alpha_2\beta_2)X$. The cooperative energy change at the intersubunit contact upon binding the first oxygen is ${}^1\Delta G_2 - {}^0\Delta G_2$.

2. Binding the Last Oxygen. Similarly, for the fourth binding step the cooperative energy change at the contact is ${}^4\Delta G_2 - {}^3\Delta G_2$, where ${}^3\Delta G_2 = \Delta G_{22} - \Delta G_{44} + {}^4\Delta G_2$. ${}^3\Delta G_2$ is defined by the unique way in which triply-liganded tetramer may be formed: $(\alpha\beta)X_2 + (\alpha\beta)X \leftrightarrows (\alpha_2\beta_2)X_3$.

3. Binding the Second and Third Oxygens. Since ${}^3\Delta G_2$ and ${}^1\Delta G_2$ (scheme [2]) can be known uniquely, the sum of the intersubunit cooperative energy changes corresponding to the two middle steps of binding can be calculated: ${}^3\Delta G_2 - {}^1\Delta G_2$. But the energy of association to doubly-liganded tetramer is *not* simply specified by the energies of scheme [2], since $(\alpha_2\beta_2)X_2$ may be formed in two ways:
symmetrically,

$$2(\alpha\beta)X \overset{{}^2K_2^S}{\leftrightarrows} (\alpha_2\beta_2)X_2, \qquad [7]$$

or asymmetrically,

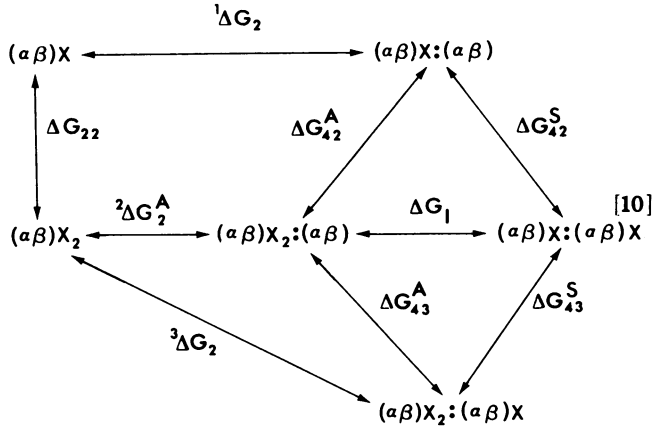
$$(\alpha\beta)X_2 + (\alpha\beta) \overset{{}^2K_2^A}{\leftrightarrows} (\alpha_2\beta_2)X_2. \qquad [8]$$

When we calculate the experimentally derivable *apparent association energy*, $\overline{{}^2\Delta G_2}$, for forming doubly-liganded tetramer, corresponding to the second oxygen binding,

$$\overline{{}^2\Delta G_2} - {}^1\Delta G_2 = \Delta G_{42} - \Delta G_{22}, \qquad [9]$$

we are faced with the question of how the energies of reactions [7] and [8] are related to the experimental quantity, $\overline{{}^2\Delta G_2}$.

To establish the relationships between intersubunit contact energies and the second and third binding steps, it is necessary to elaborate scheme [2] in greater detail. The two modes of dissociation and of binding by the doubly-liganded tetramer are depicted below.



The tetramers are represented as dimer pairs with a colon denoting the contact region. For example, the singly-liganded tetramer $(\alpha\beta)X:(\alpha\beta)$ represents all tetrameric species having one ligand bound. There is no distinction between states in which the bound ligand is distributed on different dimer pairs, i.e., $(\alpha^1\beta^1)X:(\alpha^2\beta^2)$ and $(\alpha^1\beta^1):(\alpha^2\beta^2)X$ are not distinguished, nor is there between states in which the ligand is bound to the α or β chain of a dimer. The species designations and corresponding energy relationships again are well-defined averages over microscopic equilibrium states. Distinction *is* made between symmetric and asymmetric forms of the doubly-liganded tetramer, $(\alpha\beta)X_2:(\alpha\beta)$ and $(\alpha\beta)X:(\alpha\beta)X$, as shown. Each energy term shown in scheme [10] is related to an equilibrium constant that bears the same notation; ${}^2\Delta G_2^A$ and ${}^2K_2^A$ represent the formation of asymmetric doubly-liganded tetramer (see *Appendix*). Using these definitions and the isomerization constant K_I representing the ratio of concentrations of the two forms of doubly-liganded tetramer, it is possible to interpret the apparent association energy $\overline{{}^2\Delta G_2}$. From Equations [9], [A-6], and [A-9].

$$\overline{{}^2K_2} = {}^1K_2k_{42}/k_{22} = {}^2K_2^A(1 + K_I) \qquad [11]$$

$$\overline{\Delta{}^2G_2} = {}^2\Delta G_2^A - RT \ln(1 + K_I) \qquad [12]$$

It is *not* possible to determine the energy terms ${}^2\Delta G_2^A$ and ${}^2\Delta G_2^S$ from the experimental quantity $\overline{{}^2\Delta G_2}$ unless K_I is known independently. The simplest case possible is that where ligand is bound noncooperatively to sites of equal binding affinity on α and β chains. Then $K_I = 4$ and Equation [12] becomes simply $\overline{{}^2\Delta G_2} = {}^2\Delta G_2^A - RT \ln 5$.

SIMULATED CASES

To explore the sensitivity of the linkage functions we have numerically simulated the behavior of several "models" which represent different ways of parcelling out the cooperative energy changes at the dimer–dimer interface upon binding. For example, it has been proposed (21) that the six interchain salt links broken in going from deoxy to oxy hemoglobin (7) have equal energy and are disrupted in a 3–2–1–0 sequence with successive oxygen binding. (The two intrachain salt links are ignored.) For a total energy difference of 8 kcal between ${}^4\Delta G_2$ and ${}^0\Delta G_2$, the successive free energies of dimerization will differ by 4, 2.67, 1.33, and 0 kcal (see Table 1). Scheme [2] is completely determined by these energies and the other entries in Table 1, so the functions $\bar{Y}$ and ${}^zK_2$ can be calculated.

The behavior of $\bar{Y}$ for this model as a function of total heme concentration is shown in Fig. 1. The binding isotherm at 10^{-4} M heme (1.6 mg/ml) appears similar in shape to a tetramer isotherm that has been shifted to slightly lower values of pO_2. We tried to analyze this isotherm in terms of four successive binding constants (Eqs. [A-2], [A-3], and [A-11]), using both a Nelder–Mead simplex procedure (22) and a pseudo-linearization of the saturation function (23). Both procedures were first shown to return the binding constants of Table 1 (model A, k_{4i}) from values of the tetramer isotherm. However the isotherm at 10^{-4} M heme produced highly unsatisfactory binding constants by both procedures. The Nelder–Mead simplex returned an infinitesimal k_{43} and an astronomical k_{44} (0.012, 0.038, 6×10^{-10}, 5×10^7); the pseudo-linearization rather consistently returned negative values for k_{43} and k_{44} (0.012, 0.053, −0.019, −1.183). As expected, the sets of derived k_{4i} are strongly dependent on the portion of the curve used in the analysis. The values mentioned above arise from attempts to fit the whole isotherm and are independent of the starting guesses used in the simplex routine. It is clear from these and similar calculations that experimentally determined binding constants do not reflect properties of tetramer alone. The differences are considerably greater than the accuracy required for reliably determining the four

TABLE 1. *Summary of simulations**

Model	Stage of oxygenation i	$({}^{i-1}\Delta G_2 - {}^{i}\Delta G_2)$†	ΔG_{4i}‡	k_{4i}§
A	1	−4.000	−4.402	0.004
	2	−2.667	−5.876	0.045
	3	−1.333	−6.135	0.071
	4	0	−7.598	0.886
B	1	−8	−0.402	4×10^{-6}
	2	0	−8.532	4.441
	3	0	−7.468	0.710
	4	0	−7.598	0.886
C	1	0	−8.402	3.549
	2	−8	−0.532	5×10^{-6}
	3	0	−7.468	0.710
	4	0	−7.598	0.886
D	1	0	−8.402	3.549
	2	0	−8.528	4.404
	3	−8	0.528	7×10^{-7}
	4	0	−7.598	0.886
E	1	0	−8.402	3.549
	2	0	−8.528	4.404
	3	0	−7.472	0.713
	4	−8	0.402	9×10^{-7}
F	1	−2	−6.402	0.113
	2	−2	−6.528	0.140
	3	−2	−5.472	0.023
	4	−2	−5.598	0.028
C′	1	0	−4.000	0.0018
	2	−8	−4.934	0.0090
	3	0	−3.066	0.0004
	4	0	−12.000	1750
E′	1	0	−4.000	0.0018
	2	0	−12.934	8754
	3	0	−3.066	0.0004
	4	−8	−4.000	0.0018

Noncooperative dimers are Models *A–F* in which $\Delta G_4 = -32$ kcal; ΔG_{22} and ΔG_{21} differ by statistical factor $-RT \ln 4$; $\Delta G_{21} = -8.402$ and $\Delta G_{22} = -7.598$. Cooperative dimers are Models *C′* and *E′*; $\Delta G_{21} = -4.000$ and $\Delta G_{22} = -12.000$.

* Simulations pertain to 20°, ${}^{0}\Delta G_2 = -15.5$ kcal.

† Cooperative energy change at dimer-dimer interface.

‡ Free energy of oxygen binding (kcal/mole of O_2 per mole of heme).

§ Sequential binding constant referred to mm Hg for O_2. See Eq. [A-2].

successive binding constants (14, 15). Assessment of the degree of subunit dissociation is always required.

Several other simulated cases are presented in Table 1. In four of them, all the cooperative energy is expended at a single binding step. In terms of allosteric theory these would correspond to an "all or none" transition from the deoxy state (T) to the oxy state (R), upon binding the appropriate ligand. The fifth is a case where the cooperative energy is equipartitioned.

Fig. 2 compares the behavior of the association constant, ${}^{z}K_2$, for these models. The *solid lines* show that for noncooperative dimer the models are easily distinguished unless there is a substantial fraction ($\geq 50\%$) of the energy expended at the first binding step, e.g., curves *A* and *B* are indistinguishable. Even in that case, the curves are found to be very sensitive to the overall cooperative energy (not shown). The *dashed curves* show the effect of cooperative binding by the dimer. Inspection of Table 1 for these two cases shows that relying solely on either ${}^{z}K_2$ or $\bar{Y}$ may lead to incorrect (and different) pictures of the energetics, whereas the combination defines the system much more exactly.

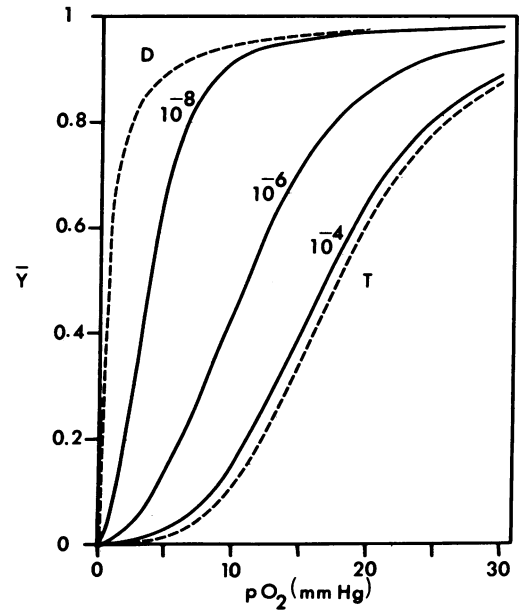


FIG. 1. Saturation function $\bar{Y}$ for case *A* in Table 1 at different values of the total molar concentration of heme (——); Saturation functions (– – –) for *D* (dimer) and *T* (tetramer). The dimer is assumed to bind noncooperatively.

DISCUSSION

We wish to emphasize that the theoretical analyses presented here do not provide any model for cooperative interactions in hemoglobin. The relationships are model independent, defining the phenomenological framework to which both experi-

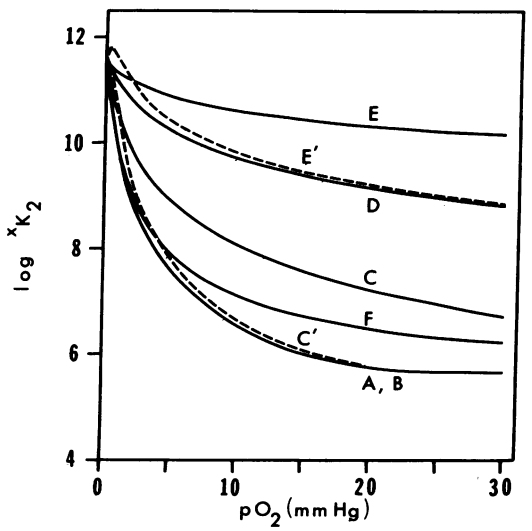


FIG. 2. Dimerization constant for the cases in Table 1. Noncooperative dimer (——); cooperative dimer, *C′* and *E′*, (– – –). Cases *A* and *B* are not identical but cannot be distinguished at this scale.

mental data and hypothetical models must conform. Indeed, the analysis provides a means of interpreting ambiguities which may arise in relating molecular models to experimental findings. For example, such ambiguities may occur if the dimer binds oxygen cooperatively (cases C′ and E′, Fig. 2 and Table 1). Neither ${}^{x}K_2$ nor $\bar{Y}$ alone will suffice to define the distribution of cooperative energy changes at the dimer–dimer interface. Both sets of data, together with this analysis, are required to resolve such a system.

Appendix

A. Binding Constants. The free energies shown in schemes [2] and [10] correspond ($\Delta G_{ni} = -RT \ln k_{ni}$) to equilibrium constants for successive binding of the ith ligand onto protein containing n subunits, according to the reaction

$$(\alpha_{n/2}\beta_{n/2})X_{i-1} + X \rightleftharpoons (\alpha_{n/2}\beta_{n/2})X_i. \qquad \text{[A-1]}$$

The sequential binding constants are:

$$k_{ni} = \frac{[(\alpha_{n/2}\beta_{n/2})X_i]}{[(\alpha_{n/2}\beta_{n/2})X_{i-1}][X_i]} \qquad \begin{matrix} i = 1, \ldots n \\ n = 2, 4 \end{matrix} \qquad \text{[A-2]}$$

A second set of experimental constants (the "Adair" constants) are more convenient in formulating the linkage relationships and binding isotherms.

$$K_{ni} = \frac{[(\alpha_{n/2}\beta_{n/2})X_i]}{[(\alpha_{n/2}\beta_{n/2})][X]^i} = \prod_1^i k_{ni} \qquad \text{[A-3]}$$

For the second binding step

$$k_{42}{}^{S} = \frac{[(\alpha\beta)X:(\alpha\beta)X]}{[(\alpha_2\beta_2)X][X]}; \qquad k_{42}{}^{A} = \frac{[(\alpha\beta)X_2:(\alpha\beta)]}{[(\alpha_2\beta_2)X][X]} \qquad \text{[A-4]}$$

For the third binding step

$$k_{43}{}^{S} = \frac{[(\alpha_2\beta_2)X_3]}{[(\alpha\beta)X:(\alpha\beta)X][X]};$$

$$k_{43}{}^{A} = \frac{[(\alpha_2\beta_2)X_3]}{[(\alpha\beta)X_2:(\alpha\beta)][X]} \qquad \text{[A-5]}$$

B. The Isomerization Constant

$$K_I = \frac{[(\alpha\beta)X:(\alpha\beta)X]}{[(\alpha\beta)X_2:(\alpha\beta)]} = \frac{k_{42}{}^{S}}{k_{42}{}^{A}} = \frac{k_{43}{}^{A}}{k_{43}{}^{S}} \qquad \text{[A-6]}$$

C. The Subunit Association Constants

$${}^{0}K_2 = \frac{[(\alpha_2\beta_2)]}{[(\alpha\beta)]^2} \qquad {}^{4}K_2 = \frac{[(\alpha_2\beta_2)X_4]}{[(\alpha\beta)X_2]} \qquad \text{[A-7]}$$

$${}^{1}K_2 = \frac{[(\alpha_2\beta_2)X]}{[(\alpha\beta)X][(\alpha\beta)]} \qquad {}^{3}K_2 = \frac{[(\alpha_2\beta_2)X_3]}{[(\alpha\beta)X_2][(\alpha\beta)X]} \qquad \text{[A-8]}$$

$${}^{2}K_2{}^{S} = \frac{[(\alpha\beta)X:(\alpha\beta)X]}{[(\alpha\beta)X]^2} \qquad {}^{2}K_2{}^{A} = \frac{[(\alpha\beta)X_2:(\alpha\beta)]}{[(\alpha\beta)X_2][(\alpha\beta)]} \qquad \text{[A-9]}$$

D. The Binding Isotherms

$$\bar{Y}_2 = \frac{K_{21}(X) + 2K_{22}(X)^2}{2[1 + K_{21}(X) + K_{22}(X)^2]} \qquad \text{[A-10]}$$

$$\bar{Y}_4 = \frac{K_{41}(X) + 2K_{42}(X)^2 + 3K_{43}(X)^3 + 4K_{44}(X)^4}{4[1 + K_{41}(X) + K_{42}(X)^2 + K_{43}(X)^3 + K_{44}(X)^4]} \qquad \text{[A-11]}$$

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