COOPERATIVE FREE ENERGIES FOR NESTED ALLOSTERIC MODELS AS APPLIED TO HUMAN HEMOGLOBIN

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ABSTRACT A model is developed for ligand binding to human hemoglobin that describes the detailed cooperative free-energies for each of the ten different ligated (cyanomet) species as observed by Smith and Ackers (Smith, F. R., and G. K. Ackers. 1985. *Proc. Natl. Acad. Sci. USA*.82:5347–5351). The approach taken here is an application of the general principle of hierarchical levels of allosteric control, or nesting, as suggested by Wyman (Wyman, J. 1972. *Curr. Top. Cell. Reg.* 6:207–223). The model is an extension of the simple two-state MWC model (Monod, J., J. Wyman, and J. P. Changeux. 1965. *J. Mol. Biol.* 12:88–118) using the idea of cooperative binding within the *T* (deoxy) form of the macromolecule, and has recently been described as a "cooperon" model (Di Cera, E. 1985. Ph.D. thesis). The *T*-state cooperative binding is described using simple interaction rules first devised by Pauling (Pauling, L. 1935. *Proc. Natl. Acad. Sci. USA.* 21:186–191). In this application three parameters suffice to describe the cooperative free-energies of the 10 ligated species of cyanomet hemoglobin. The redox process in the presence of cyanide, represented as a Hill plot, is simulated from Smith and Ackers' cooperative free-energies and is compared with available electrochemical binding measurements.

INTRODUCTION

The general study of thermodynamic properties of hemoglobin (Hb) intermediates at various specific states of oxidation offers potential insight into the mechanism of cooperative binding that is not possible from the examination of properties averaged over a wider distribution of species. For example, oxygen binding to partially oxidized hybrids of trout I Hb afforded useful information in detailed modeling of that system (1). Recently, Smith and Ackers (2) have evaluated the cooperative free-energy of the ten specifically ligated (cyanomet) species of human hemoglobin, HbA°. Although cyanomet ligation is not exactly equivalent to reversible oxygen-binding, the specific thermodynamic information obtained for these species provides us with an unprecedented degree of detail about the distribution of cooperative free-energies among them.

The cooperative free-energy measured by Smith and Ackers (2) was determined by the difference between the free-energies for dimer → tetramer association of each isolated, specifically ligated species and of the unligated form. By a thermodynamic cycle this is equivalent to the difference in the binding free-energy between a specific tetramer and the noncooperative dimers. Most current thermodynamic models describing cooperative binding have been formulated to account for the Adair constants, which do not acknowledge differences between configurations having the same number of ligands. The detailed, configurational species information now available from the Smith and Ackers study provides a much more stringent test for any proposed model.

In this paper we examine the predictions of the simple MWC model applied to Hb, finding it to account for most of the observed features, but to be inadequate in some respects. Those results suggest the inclusion of an internal positive or negative cooperativity within one of the allosteric forms of the MWC model such as has been used in the cooperon model (3, 4). A similar approach was used by King (5) to explain the cooperativity of cyanide binding in complexes of nickel and zinc ions. Application of two-state modeling to the internal cooperativity has been found appropriate in the case of oxygen binding to tarantula hemocyanin, a 24-binding-site protein (6). All these treatments may be considered to represent different aspects of the nesting idea as conceived by Wyman (7). We show that with simple rules, like those introduced by Pauling (8), describing interactions between subunits in the *T* form, it is

possible to describe all the presently known features of cooperative free-energies of cyanomet Hb derivatives.

Hemoglobin redox reactions, of which cyanomet ligation is an example, may also be represented in the form of an "electron binding" curve (fractional oxidation vs. oxidation potential) and are illustrated with data obtained some years ago (9). This process, studied in the absence of cyanide, appears to be similar in the extent of cooperativity but different in detail from the binding processes examined by Smith and Ackers.

THEORY

For a given number i of ligands bound to the Hb tetramer, there may be a number of different configurations with respect to disposition of the ligands, which shall be referenced by the letter j. For human hemoglobin there are 10 uniquely ligated species, which are shown schematically in Table I. The cooperative free-energy for a given species is defined as the difference between the overall binding free-energy for the ijth species of the native Hb tetramer and the overall binding free-energy for the ith species of the noncooperative Hb dimers

$$-\Delta G_{ij}\,(\text{coop}) = RT \ln \beta^*_{ij} - RT \ln \kappa_X^i, \qquad (1)$$

where β^*_{ij} is the overall binding constant without statistical factors (g_{ij}) for the reaction $\text{Hb} + iX \rightleftharpoons \text{Hb}X_{ij}$ with ligand X, and κ_X is the intrinsic binding constant for X to the noncooperative Hb dimer. The cooperative free-energy has not been determined in full detail for oxygen binding, due to the difficulty of isolating the unstable individual species. However, the cooperative free-energies of specifically oxidized cyanide-ligated species, because of their greater stability, have been measured by Smith and Ackers and are shown in the first column of Table I.

We shall represent this oxidation, at a given cyanide activity, by $\text{Hb} \rightarrow \text{Hb}_{ij}^{+i} + ie^-$ in which Hb is the fully reduced form and ie^- are the electrons released leaving the hemoglobin with i additional + charges. As before, the subscript j denotes a particular configuration of the oxidized sites. The observed oxidation potential for this reaction may then be expressed as $E_{ox} = -RT/F \ln(a_e)$, where F is the Faraday constant and a_e is the electron activity as defined against the standard hydrogen electrode. The oxidation process is described by the sum of concentrations of the various oxidized species as represented by the oxidation polynomial, P_{ox}. The oxidation polynomial for the four-site reaction is[1]

$$P_{ox} = 1 + \beta_1(1/a_e) + \beta_2(1/a_e)^2 + \cdots + \beta_4(1/a_e)^4 \qquad (2)$$

where

$$\beta_i = \sum_j g_{ij}\,\beta^*_{ij}.$$

Each term in the polynomial represents the concentration of an individual species ij relative to the fully reduced species (10, 11).

The model formulation of the binding constant for a particular species ij (β^*_{ij}) is conveniently written down from the binding polynomial specified by that model: each β^*_{ij} is the coefficient for any i-power term corresponding to configuration j. The Adair constants β_i can also be calculated by summing over j the β^*_{ij} weighted by the degeneracy g_{ij}. In order for one to arrive at values of the cooperative free-energy specified by the model, it is necessary to assign a value to κ_X. In the case of oxygen binding to HbA, it has been observed that the intrinsic binding constant for the dimer is essentially the same as the last intrinsic binding constant to the tetramer (12). In this paper we shall assume the same relation holds for the cyanomet derivatives as well.

To relate the results of cooperative free-energy determinations to actual redox experiments, it is useful to express the fractional oxidation in terms of the Adair constants and the oxidation potential. The extent of oxidation is given by the logarithmic derivative of P_{ox} with respect to the oxidation potential. The fractional oxidation (θ) for the four-electron binding process is then

$$4\theta = \frac{\partial \ln P_{ox}}{\partial E_{ox}} = \frac{\beta_1 \exp\left(\frac{E_{ox}F}{RT}\right) + 2\beta_2 \exp\left(\frac{2E_{ox}F}{RT}\right) + \cdots}{1 + \beta_1 \exp\left(\frac{E_{ox}F}{RT}\right) + \beta_2 \exp\left(\frac{2E_{ox}F}{RT}\right) + \cdots}. \qquad (3)$$

The β_i here are defined for a specific cyanide activity, in particular 10 μM as used by Smith and Ackers. In principle one could study the fractional oxidation as a function of oxidation potential at that cyanide activity, but such studies have not yet been carried out. In this paper we shall use Eq. 3 to simulate Hill plots for such a process using the β_i's predicted by the Smith and Ackers data.

RESULTS AND DISCUSSION

For the simple MWC model as applied to the Hb tetramer, the binding polynomial has the form

$$P(\text{MWC}) = \frac{1}{1+L}(1 + \kappa_R x)^4 + \frac{L}{1+L}(1 + \kappa_T x)^4, \qquad (4)$$

in which κ_R and κ_T are the intrinsic binding constants for the appropriate ligand X with activity x to the high-affinity and low-affinity conformations of the macromolecule, and L is the equilibrium constant for the transition between these two conformations when no ligand is present. Expanding P_{MWC} the β^*_{ij} are seen to have the form

$$\beta^*_{ij}\,(\text{MWC}) = \kappa_R^i \left(\frac{1 + Lc^i}{1 + L}\right). \qquad (5)$$

Here c is defined as the ratio of κ_T/κ_R. In the simplest MWC model all configurations of i ligands bound to the macromolecule are energetically equivalent, so that all $\beta^*_{ij} = \beta^*_i$. By substituting Eq. 5 into Eq. 1 and allowing $\kappa_X = \kappa_R$ we obtain

$$\Delta G_i(\text{coop, MWC}) = -RT \ln \left(\frac{1 + Lc^i}{1 + L}\right). \qquad (6)$$

For a symmetrical binding curve $Lc^2 = 1$ for a tetrameric macromolecule. With a typical L value and assuming for convenience symmetrical binding, we obtain the values shown in the second column of Table I. The cooperative free-energies predicted by the MWC model clearly demonstrate the three equally spaced free-energy levels discovered by Ackers. The leveling-off of the cooperative free-energies and their consequent equivalence at the third and fourth ligation steps in this model results from the essentially complete transition of the macromolecules to the R form by the third step of ligation. This fact is indicated by the logarithmic argument of the cooperative

[1]Here the specific effects of cyanide ion are implicitly included in the β's. For example $\beta_{ij} = \beta^o_{ij}\,[P_{ij}(y)/P_o(y)]$ where the $P(y)$ are the appropriate cyanide binding polynomials (y is cyanide activity) for the ijth and reference species.

free-energy expression, given at the left in the column, which is seen to be on the order of $1/(1 + L)$ for the third and fourth ligation states. The model is, however, unable to account for the two different cooperative free-energies associated with doubly ligated Hb when the molecule is ligated either on both types of chains (species 21 and 22 in Table I) or on just one type of chain (species 23 and 24). To explain this splitting, a model must distinguish between these configurations, as the simple MWC does not.

Since the majority of Smith and Ackers' observations are nevertheless explainable in terms of the simple MWC model, it seemed likely to us that a simple modification would yield all of the observed cooperative free-energies. By allowing one or both of the MWC allosteric states to individually possess cooperativity, according to the cooperon idea (3), we introduce a way of describing differences in single ligation-state energy levels.

For human Hb, the simplest version (4) of the generalized cooperon model (3) proposes the cooperative substructures to be $\alpha\beta$ dimers, although other subunit pairs could be chosen within the tetramer and yield similar results. The binding polynomial for the $\alpha_1\beta_1$ dimer (where the α and β chains are considered identical) nested in the T form of an MWC-type model is

$$P_{\alpha_1\beta_1} = 1 + (\kappa_T^{\alpha_1} + \kappa_T^{\beta_1})\, x + \delta\, \kappa_T^{\alpha_1} \kappa_T^{\beta_1} x^2, \qquad (7)$$

where the κ_T are the binding constants for the α_1 or β_1 subunits of the macromolecule in the low-affinity (T) form and δ is an interaction parameter with a value <1 when the dimer is negatively cooperative and >1 when the dimer is positively cooperative. The binding polynomial for the $\alpha_2\beta_2$ dimer has the same form as Eq. 7. In the R state the dimers are assumed to be noncooperative so that the binding polynomial P for a tetramer modeled with two dimeric cooperons in the T state is then

$$P(\text{dimeric cooperon}) = \frac{1}{1 + L}(1 + \kappa_R x)^4 + \frac{L}{1 + L}(P_{\alpha_1\beta_1} \cdot P_{\alpha_2\beta_2}). \qquad (8)$$

Again the β_{ij}^* are found from the expansion of Eq. 8, grouping terms by the specifically ligated subunits. All subunits are assumed for simplicity to have identical intrinsic affinities in the T state, and c is again the ratio κ_T/κ_R. The cooperative free-energies for this model are shown in the column of Table I designated "Dimeric Cooperon". Values of δ and c were deduced by inspection to provide the agreement shown in the table. As in the MWC model the logarithmic argument is on the order of $1/(1 + L)$ at the third and fourth ligation steps. Also the doubly ligated tetramers in which both ligands are on the same dimer have the same energy as the singly ligated species, and the $\alpha\alpha$- and $\beta\beta$-ligated species are at the level of the triply and quadruply ligated species, in accordance with the results of Smith and Ackers shown in column 1. However those experimental observations show that all mixed-chain doubly ligated species (i.e., species of the type 21 and 22) have the same cooperative free energy, regardless of whether the ligated subunits are part of the same $\alpha\beta$ dimer or not. The dimeric cooperon model is inadequate in that it assigns different cooperative free-energies to these species. A proper model requires the use of a cooperative scheme that does not specify individual subunit pairs in the T form.

A nested interaction involving more than two sites, or in the terms of the cooperon idea (3), a cooperon "valency" higher than two, quantitatively accounts for the observations of Smith and Ackers by introducing the flexibility of the Pauling (8) or KNF (13) approach. This requires the formulation of a consistent set of rules that apply to the changes of interactions between subunits upon ligation of one or more of them. A feasible scheme is indicated in Fig. 1. A tetrameric cooperon in the T form of the macromolecule is depicted by a square arrangement of subunits interacting along the diagonals and the sides. This arrangement recalls the I_2H_4 subunit interaction scheme for tetramers with isologous ($\alpha\alpha$ or $\beta\beta$) and heterologous ($\alpha\beta$) interactions, which was proposed by Cornish-Bowden and Koshland (14) as an extension of the KNF tetrahedral interaction scheme (13). The binding polynomial $P_{I_2H_4}$ for the T-form tetramer is the sum of the terms representing the relative populations shown beneath each species. A simplifying assumption proves appropriate in allowing all interactions to be of correlated strength, i.e., $\delta^{-1} = \gamma$ (symbols are explained in Fig. 1). The binding polynomial for the Hb molecule modeled with a noncooperative R form and the I_2H_4 cooperon for the T form is then

$$P(I_2H_4 \text{ cooperon}) = \frac{1}{1 + L}(1 + \kappa_R x)^4 + \frac{L}{1 + L}(P_{I_2H_4}). \qquad (9)$$

The β_{ij}^* relative to the R-state (noncooperative) binding

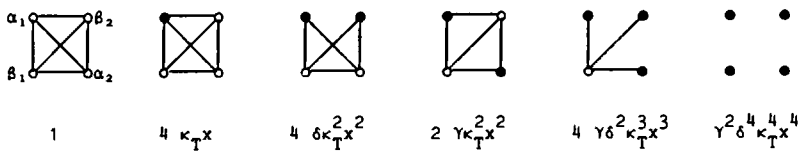

FIGURE 1 Schematic representation of subunit interactions in an I_2H_4 cooperon nested in the T-state of a four-site macromolecule. Filled circles indicate ligated subunits. The unligated macromolecule has diagonal interactions of strength RTlnγ and side interactions of strength $RT \ln \delta$. These interactions are broken upon ligation of connected subunits. Pairs of subunits on the sides of the square ($\alpha\beta$ pairs) are positively cooperative whereas subunits connected diagonally ($\alpha\alpha$, $\beta\beta$ pairs) are negatively cooperative.

TABLE I

COMPARISON OF COOPERATIVE FREE ENERGIES FOR SELECTED MODELS

COOPERATIVE FREE ENERGIES (KCAL/MOLE-TETRAMER)

			Experiment‡	MWC		Dimeric Cooperon		I_2H_4 Cooperon	
ij	g_{ij}	Species	ΔG_{coop}	β^*_{ij}/κ_R^i	ΔG_{coop}§	β^*_{ij}/κ_R^i	ΔG_{coop}‖	β^*_{ij}/κ_R^i	ΔG_{coop}¶
00	1	α_1 β_2 / β_1 α_2	0	1	0	1	0	1	0
11	2	X /	2.9	$\frac{1 + Lc}{1 + L}$	3.1	$\frac{1 + Lc}{1 + L}$	3.1	$\frac{1 + Lc}{1 + L}$	3.1
12	2	/ X	3.2	$\frac{1 + Lc}{1 + L}$	3.1	$\frac{1 + Lc}{1 + L}$	3.1	$\frac{1 + Lc}{1 + L}$	3.1
21	2	X / X	3.0	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2\delta}{1 + L}$	3.1	$\frac{1 + Lc^2\delta}{1 + L}$	3.1
22	2	X X /	2.7	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2\delta}{1 + L}$	3.1
23	1	X / X	6.2	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2\gamma}{1 + L}$	6.2
24	1	X / X	5.9	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2}{1 + L}$	5.8	$\frac{1 + Lc^2\gamma}{1 + L}$	6.2
31	2	X X / X	5.8	$\frac{1 + Lc^3}{1 + L}$	6.2	$\frac{1 + Lc^3\delta}{1 + L}$	5.8	$\frac{1 + Lc^3\gamma\delta^2}{1 + L}$	5.8
32	2	X / X X	6.0	$\frac{1 + Lc^3}{1 + L}$	6.2	$\frac{1 + Lc^3\delta}{1 + L}$	5.8	$\frac{1 + Lc^3\gamma\delta^2}{1 + L}$	5.8
41	1	X X / X X	5.9	$\frac{1 + Lc^4}{1 + L}$	6.2	$\frac{1 + Lc^4\delta^2}{1 + L}$	5.8	$\frac{1 + Lc^4\gamma^2\delta^4}{1 + L}$	5.8

‡Values taken from Smith and Ackers (2).

§MWC parameters: $L = 36{,}000$ and $c = 0.0053 = L^{-1/2}$.

‖Dimeric cooperon parameters: $L = 36{,}000$, $c = L^{-1/2}$, and $\delta = 1/c$.

¶I_2H_4 cooperon parameters: $L = 36{,}000$, $c = L^{-1/2}$, $\delta = 1/c$, and $\gamma = c$.

can be obtained as demonstrated in the previous cases and are shown at left in the third column of Table I. By inspection, we have chosen L, c, and δ such that the resulting cooperative free-energies are virtually identical to those determined experimentally by Smith and Ackers. It is possible that other simple interaction schemes for the T form can be devised that will lead to equally good agreement. It should be pointed out, however, that it appears that any of these would be inadequate without the underlying allosteric equilibria.

Each of the models that have been considered here can be used to generate Hill plots of the oxidation process.

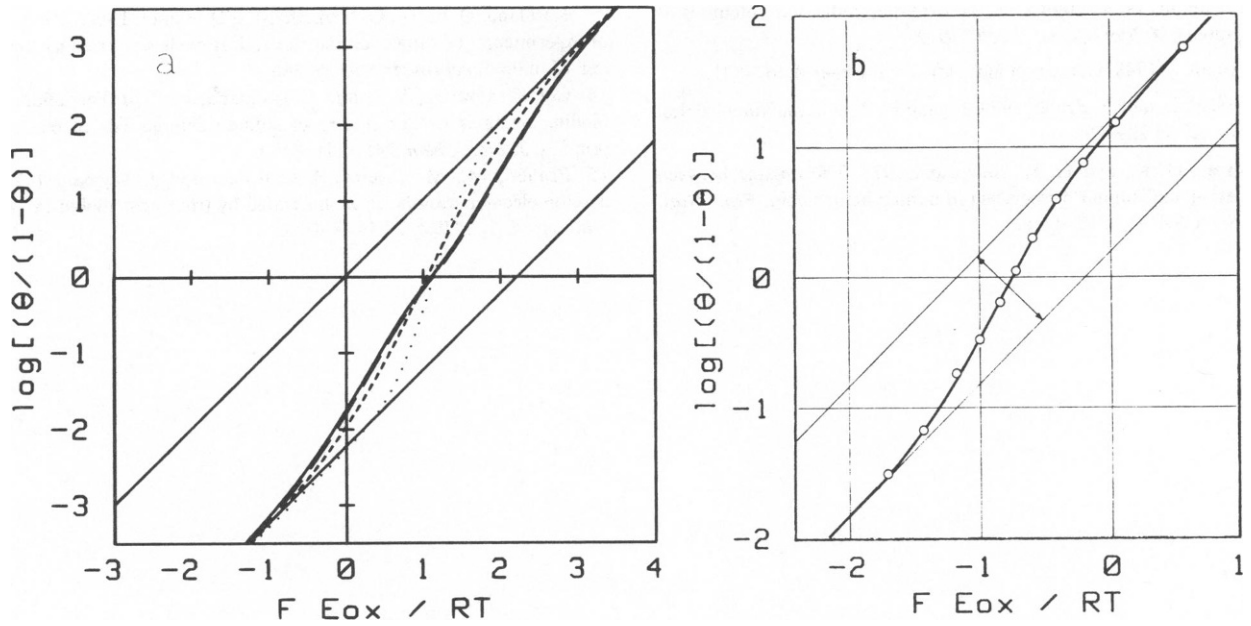

FIGURE 2 Hill plots of oxidation processes in human Hemoglobin. (*a*) Solid line (—) is simulation of oxidation in the presence of 10 μM cyanide, pH 7.4, 22°C from the cooperative free-energies determined by Smith and Ackers (2); (· · · ·), MWC model prediction; (---), dimeric cooperon model; (— — —), I_2H_4 cooperon model. Simulations shown here use constants as given at the bottom of Table I. (*b*) Oxidation data obtained by Antonini et al. (9) in the absence of cyanide, pH 8.65, 30°C.

These are shown in Fig. 2 *a*. The theoretical line representing the I_2H_4 cooperon model is coincident with that obtained from the data of Smith and Ackers.

As noted above, the cooperative free-energies for any model lead directly to the binding polynomial and to the fractional degree of oxidation in an equilibrium solution as a function of oxidation potential. Measurements of this quantity for HbA° have been performed (9), although in the absence of cyanide, and a Hill plot of the binding process is shown in Fig 2 *b*. There is a similarity in the degree of cooperativity for both curves, but the presence of multiple inflection points is not immediately evident in Fig. 2 *b* and may reflect the difference between oxidation in the presence and absence of cyanide. Complex binding shapes of this sort have been observed in the case of reversible oxygen binding to Trout IV hemoglobin at low pH, but these observations have been explained by simple heterogeneity of binding sites (15). In any case the redox process in the presence of cyanide appears to be quite different from the physiological process of oxygen binding to human hemoglobin.

In summary we have demonstrated that a simple extension of the allosteric MWC model, incorporating cooperative interactions in the *T* the state as described by an I_2H_4 cooperon, can account for the detailed cooperative free-energies of the ten cyanomet ligation states for HbA° as observed by Smith and Ackers (2). We have also shown how these cooperative free-energies can be used to obtain oxidation polynomials and Hill plots for the oxidation process. Comparison of these Hill plots to those determined electrochemically for hemoglobin yield general agreement in overall cooperativity but differences in detailed shape, presumably due specific effects of cyanide binding.

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