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Analysis of hemoglobin oxygenation from combined equilibrium and kinetic data

Is quaternary enhancement necessary?

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An experimental approach based on four independent techniques, in which kinetic and equilibrium measurements of subunit assembly reactions are combined with concentration-dependent oxygen-binding curves, has previously been used to resolve parameters of the linkage system for human hemoglobin over a wide range of conditions [(G.K. Ackers and H.R. Halvorson, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 4312; F.C. Mills et al., Biochemistry 15 (1976) 1093; M.L. Johnson et al., Biochemistry 15 (1976) 5363). Throughout this extensive body of results it has been found that the affinity for binding oxygen to tetramers at the fourth step exceeds the mean affinity of dissociated dimers. The existence of this "quaternary enhancement" effect has recently been questioned by Gibson and Edelstein (J. Biol. Chem. 262 (1987) 516) and by Philo and Lary (J. Biol. Chem. 265 (1990) 139) on the basis of kinetically derived oxygen-binding constants that do not exhibit quaternary enhancement. These authors have also suggested that quaternary enhancement might not be necessary to explain the oxygen-binding data mentioned above. In this study, we have explored the effect of constraining the numerical analysis of oxygen-binding data against the new kinetically derived binding constants. It is found that the sets of linkage constants which are compatible with both the oxygen-binding data and the new kinetically derived dimer binding constant require both quaternary enhancement and substantial dimer cooperativity. Increasing the dimer cooperativity to compensate completely for quaternary enhancement requires both dimeric and tetrameric binding constants that disagree with the kinetically derived values. Thus, the quaternary enhancement effect cannot be eliminated by readjustment of the remaining constants of the linkage system. Possible sources of the discrepancy between the kinetically derived binding constants and the otherwise self-consistent data from the other four techniques are discussed.

1. Introduction

In tetrameric human hemoglobin the total regulatory energetics of oxygenation consists of only a few net kilocalories (i.e., up to about 8 kcal in the cooperative free energy, depending on conditions) [1–3]. The problem of experimentally resolving the energetic components of this complex regulatory mechanism is, therefore, a formidable task which has occupied investigators for decades. A major strategy has been the measurement of oxygen-binding isotherms and their numerical analysis into the four tetrameric stepwise binding free energies. Such values, obtained over a wide range of conditions, involving various regulatory effectors (i.e., H^+, Cl^-, diphosphoglycerate, CO_2), can provide a predictive framework for understanding the intermolecular interactions and correlating them with the intramolecular structure changes to which they are coupled.

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The determination of accurate oxygen-binding curves and their quantitative interpretation have posed difficult and sometimes controversial issues due to: (a) the extreme sensitivity of binding constants to binding curve shape, requiring experimental accuracies of better than 1%; (b) the high statistical correlation between binding constants (particularly the middle two), which places severe demands on the numerical analysis problem; (c) the difficulties of accurate, appropriately weighted end-point evaluation; and (d) the presence of even small amounts of dissociated dimers that can exacerbate the first three problems and provide additional errors in the resolved apparent tetramer constants.

Because of these difficulties, it is desirable to combine data from oxygen-binding equilibria with independently determined information whenever possible. Our approach to this problem [1–4] has been to combine information from four separate experimental techniques (table 1) which measure different aspects of the reaction equilibria (including kinetic components) under the premise that no single technique (e.g., measurement of oxygen-binding curves alone) would necessarily have sufficient accuracy and freedom from small, systematic errors. Only if a variety of techniques which probe different 'coordinates' of the same molecular behavior can be found to converge to a common 'answer' within a specified region of parameter space do we ascribe physical meaning to the derived results. A second feature of this strategy consists of studies by all four techniques over a wide range of solution conditions under the premise that significant effects will exhibit self-consistency in the observed trends while highly restricted conditions may produce less typical manifestations of the system's characteristic features. A third strategic component has been to take advantage explicitly of the subunit dissociation equilibria as a major coordinate for resolving both tetrameric and dimeric properties as a function of oxygenation. Not only does this provide a second dimension of experimental resolution (improving the problem of parameter correlation), but also much structural and other information indicates that cooperativity derives largely from the ligand-linked dimer-dimer interactions within the tetramer. Since these interactions are eliminated by the dissociation reactions, the energetics of subunit dissociation at different stages of oxygenation are of mechanistic interest in their own right. The pertinent relationships are summarized by the linkage scheme of fig. 1.

Experimental and computational methods developed to resolve the 11 constituent free energies (seven of which are 'independent') have been de-

Table 1

Experimental techniques used to resolve parameters of the hemoglobin linkage system [a]

Technique	Information obtained	References
(1) Stopped-flow kinetics	dimer-tetramer association rate constants (unligated)	4,17–19
(2) Haptoglobin kinetics	tetramer-dimer dissociation rate constants (unligated)	4,17,18
(3) Analytical gel permeation chromatography	dimer-tetramer association equilibrium constants (ligated)	20–22
(4) Oxygen-binding measurements	binding isotherms at each hemoglobin concentration	2,7–9

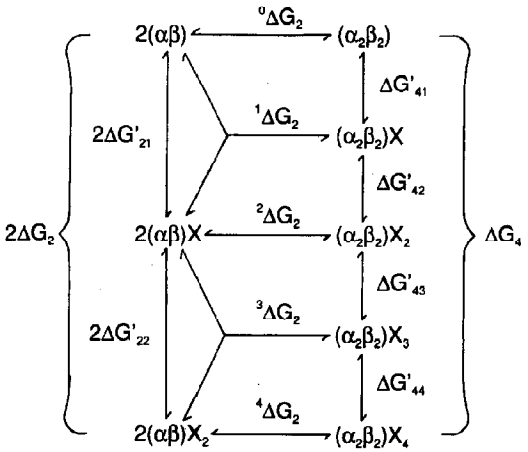


Fig. 1. Linkage scheme for the coupled processes of oxygen binding and subunit assembly. Free energy symbols represent intrinsic values (corrected for statistical factors) corresponding to the reactions taken from left to right and top to bottom. ΔG_4 represents the sum of all four stepwise free energies for tetramer oxygen binding and ΔG_2 represents the total free energy (over both steps) for oxygenating dimers.

scribed in detail [3–6]. Resolution of the complete linkage system was initially achieved with five separate data sets at pH 7.4 and 21.5 °C, which also made use of different hemoglobin preparations [2,7,8]. Subsequently the linkage system was solved [9] at four additional temperatures over the range 10–37 °C (all at pH 7.4). A third series of studies yielded complete sets of the linkage parameters at four additional pH values [8]. Parallel studies were carried out on the oxygenation of isolated α- and β-subunits and their linkage to subunit assembly [9–12], leading to the discovery of quaternary enhancement in β_4 tetramers [15] and its subsequent confirmation by Kurz and Bauer [31].

A significant feature throughout this extensive body of equilibrium and kinetic data is the finding that the affinity for binding oxygen to tetramers at the fourth step exceeds that for binding to the isolated α- and β-subunits and also for the mean binding affinity to the dissociated $\alpha\beta$ dimers. This 'quaternary enhancement' effect is illustrated by the values of table 2. It is seen that while the mean affinity of dimers ($\Delta G_2/2$) is very close to the average of values determined independently for the isolated chains (α_2 and β_4), the Gibbs energy for binding the fourth oxygen to tetramers is significantly enhanced. The average free energy of enhancement is approx. 0.9 kcal. At 21.5 °C the quaternary enhancement of 0.8 kcal corresponds to a 4-fold increase in binding constant. Another manifestation of this same effect is a higher affinity of dimers for each other when they assemble to form fully ligated tetramers as compared with the assembly of triligated tetramers, i.e., the value of $({}^3K_2/{}^4K_2)$ is less than unity (fig. 1). Quaternary enhancement is also manifested by the oxygen-binding equilibrium data of Imai and Yonetani [13] at temperatures between 10 and 35 °C, and by the binding data of Di Cera et al. [14] using a differential thin film binding technique. Direct measurement of ${}^3K_2$ and ${}^4K_2$ in cobalt-substituted hemoglobins [28] has recently provided a clearcut demonstration of quaternary enhancement which is entirely independent of binding-curve analysis. These and other studies indicating differences between triply ligated and fully ligated tetramers [29] are consistent with the observed energetics of quaternary enhancement. The existence of this effect has significant implications for understanding the hemoglobin mechanism [6,30].

Recent studies by Gibson and Edelstein [15] and by Philo and Lary [16] have provided new kinetically derived equilibrium constants for the last step of oxygen binding to tetramers (k'_{44}) and to dissociated dimers (k'_{22}). Values obtained under a particular set of conditions have led these authors to suggest that quaternary enhancement in tetramers relative to dimers might not exist. Gibson and Edelstein [15] have made specific proposals for re-analysis of the data of Mills and Ackers [9] that might accommodate the kinetically derived tetramer binding constant while eliminating significant quaternary enhancement. We have, therefore, in this study explored the effects of constraining the numerical analysis of the oxygenation data against these new constants, in order to assess their effect on evaluation of the remaining

Table 2

Quaternary enhancement in the Gibbs energies of oxygen binding to human hemoglobin

T (°C)	Isolated subunits [a]			Dimers [b]	Tetramers [c]
	$\Delta G_{\alpha 2}$	$\Delta G_{\beta 4}$	$1/2(\Delta G_{\alpha 2} + \Delta G_{\beta 4})$	$(\Delta G_2/2)$	$(\Delta G'_{44})$
21.5	-8.11 ± 0.06	-8.65 ± 0.08	-8.34 ± 0.1	-8.34 ± 0.1	-9.15 ± 0.2
33	-7.90 ± 0.06	-8.19 ± 0.04	-8.05 ± 0.1	-7.99 ± 0.1	-9.05 ± 0.2
37	-7.76 ± 0.07	-8.05 ± 0.04	-7.91 ± 0.1	-7.84 ± 0.1	-8.71 ± 0.2

[a] From oxygen-binding isotherms [9,31] and equilibrium gel permeation data [10]. The affinities listed pertain to dimeric α-subunits and tetrameric β-subunits.

[b] From medians of oxygen-binding isotherms analyzed by eq. 2, in combination with free energies of assembly ${}^0\Delta G_2$ and ${}^4\Delta G_2$.

[c] From analysis of oxygen-binding curves in combination with independently determined values of ${}^0K_2$, K_2 and K_4, and assuming noncooperative dimers.

parameters of the linkage system and to test the proposals of Gibson and Edelstein regarding data analysis. Here we describe the results of those studies, using the 13 previously published data sets referred to above, covering five temperatures at pH 7.4 and five pH values at 21.5°C. Tabulations of all the oxygen-binding data sets at pH 7.4 (106 pages) have been published as a microfiche supplement to ref. 8. Tabulations for the data at temperatures other than 21.5°C can be found in ref. 37.

2. Methods and strategy

Many of the experimental and computational methods employed in the present work have been described in detail elsewhere [1–5]. Here we briefly summarize certain aspects of these methods in order to focus on the issues at hand and to illustrate the strategies we used in arriving at the conclusions to be presented in this paper. The central strategic elements delineated in this section are: (a) values of the stepwise tetramer constants are highly constrained by their thermodynamic linkage to the subunit dissociation reactions; this can be taken advantage of to obtain additional resolving power not available from the oxygen-binding curves alone. (b) Utilizing the independent determinations of some reaction constants, the model-independent formulation of the problem reduces the unknowns to a small number of fitting parameters, usually only three. In certain cases, such as with the MWC allosteric model [30], the number of unconstrained fitting parameters can be reduced to only one (for the simplest model) or to two (for the more complex model).

2.1. Strategy for resolving the hemoglobin linkage system

The linkage scheme of fig. 1 depicts 11 equilibrium reactions among the eight stoichiometric

Table 3

Thermodynamic constants for oxygenation of hemoglobin tetramers and dimers at pH 7.4 and 21.5°C

Parameters	Values	Methods	Reference
(A) Tetramers – reported values for fourth binding step			
$\Delta G'_{44}$	-8.62 ± 0.10	kinetic	15
	-8.72 ± 0.02	kinetic	16
	-8.65 ± 0.12	combined equilibrium	2
	-9.15 ± 0.35	and kinetic	8
	-9.01	equilibrium	13
	-9.52	equilibrium	14
(B) Dimers – reported values			
ΔG_{α}^{exp}	-8.82 ± 0.06		
ΔG_{β}^{exp}	-8.49 ± 0.06	kinetic	15
$(\Delta G_{\alpha}^{exp}+\Delta G_{\beta}^{exp})$	-17.31 ± 0.08		
ΔG_2	-16.68 ± 0.05	combined equilibrium and kinetic	7
(C) Analysis of dimer cooperativity			
$\Delta G'_{\alpha}$	-8.18 ± 0.07	combined equilibrium	this study
$\Delta G'_{\beta}$	-7.85 ± 0.07	and kinetic	
$\Delta G'_{22}$	-8.64 ± 0.10		
$\Delta G'_{21}$	-8.04 ± 0.10		
$k'_{\alpha\beta}$	3.02		
K_{coop2}	0.600		

species. These 11 equilibrium constants (or the corresponding Gibbs free energies ($\Delta G = -RT \ln K$)) are defined by a smaller basis set consisting of seven constants, or combinations thereof, and these can be selected in a variety of ways. A useful strategy for choosing parameters, developed from extensive numerical explorations [2,3], consists of the following steps:

(1) The equilibrium constant ${}^0K_2$ for assembly of unligated tetramers from unligated dimers is determined by kinetic studies of the forward and reverse reactions [4,17–19]. Illustrative results of these determinations are shown in table 4 (part A) at a series of pH values. The validity of these equilibrium constants has been established by means of control experiments that account for the amplitudes as well as the rate parameters [17], and by direct equilibrium measurements [4].

(2) The equilibrium constant ${}^4K_2$ for assembly of fully oxygenated tetramers is determined by analytical gel permeation chromatography using the 'large zone' technique [20–23], or by stopped-flow kinetics [4,19] in which the amplitudes of the rate processes are shown to vary precisely according to the proportions of dimer predicted from the equilibrium ${}^4K_2$ values. Table 4 (part B) gives a representative series of the free energies at pH values corresponding to those of part A. A recently reported set of values derived from highly precise osmotic pressure measurements [23] under the same conditions is also listed for comparison. The two techniques agree to within 0.1 kcal at each pH value. The importance of ${}^4K_2$ as a critical constraint on interpretation of the linked oxygen-binding curves will be demonstrated later in this paper (section 3.2).

(3) Oxygen-binding isotherms are determined at a series of hemoglobin concentrations. The experimental system developed by Imai [24] is particularly suitable for adaptation to this purpose [2,8]. Oxygen-binding curves are measured with a precision of $\pm 0.5\%$ or better over a concentration range from less than 1 μM to several hundred μM [2].

(4) For each binding curve ($\bar{Y}$ vs molar O_2 concentration [X]) corresponding to a particular he-

Table 4

Kinetic and equilibrium contributions to dimer-tetramer assembly constants of the linkage system

pH	k_f ($M^{-1}\,s^{-1}$) ($\times 10^5$)	k_r (s^{-1}) ($\times 10^5$)	${}^0K_2$ (M^{-1}) ($\times 10^{10}$)	${}^0\Delta G_2$ (kcal)
(A) Kinetically derived values for unligated molecules [a]				
6.50	8.0 ±0.7	35.0 ±4.0	23.0 ±3.0	−15.3±0.1
7.40	11.0 ±1.0	2.2 ±0.1	5.0 ±0.7	−14.4±0.1
8.00	11.0 ±1.0	4.5 ±0.1	2.4 ±0.3	−13.9±0.1
8.50	8.2 ±0.2	0.12±0.01	0.69 ±0.03	−13.2±0.1
8.95	7.6 ±0.8	0.53±0.06	0.14 ±0.05	−12.3±0.2
9.50	4.4 ±0.4	0.95±0.01	0.047±0.005	−11.6±0.1

pH	Gel chromatography [a]	Osmotic pressure [b]
(B) Equilibrium values of ${}^4\Delta G_2$ for oxyhemoglobin (kcal)		
6.50	−7.02±0.1	–
7.40	−8.05±0.1	−7.98±0.18
8.00	−8.93±0.1	−8.77±0.05
8.50	−9.25±0.1	−9.20±0.13
8.95	−9.07±0.1	−8.84±0.13
9.50	−8.58±0.1	–

[a] Refs 4 and 15.

[b] Ref. 23; concentration scale converted to mol dimer per l.

Table 5

Median oxygen concentrations from binding curves as a function of hemoglobin concentration

Conditions were: 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM Na_2-EDTA, pH 8.5 at 21.5°C.

$[P_t]$ (M) ($\times 10^6$)	No. of data points	Median, $[\bar{X}]$ (M) ($\times 10^6$)
2.04	73	2.59 ± 0.003
2.15	69	2.60 ± 0.002
4.07	62	2.68 ± 0.003
4.46	54	2.70 ± 0.002
4.62	50	2.70 ± 0.003
7.05	59	2.73 ± 0.004
8.98	57	2.73 ± 0.005
10.15	61	2.76 ± 0.002
13.21	58	2.80 ± 0.004
25.41	63	2.83 ± 0.004
30.64	70	2.83 ± 0.003
31.48	68	2.85 ± 0.004
74.26	68	2.89 ± 0.005
85.08	71	2.87 ± 0.005

$K_4 = 1.366 \pm 0.005 \times 10^{22}/4$ mol O_2
$K_2 = 3.618 \pm 0.007 \times 10^{12}/2$ mol O_2
$\Delta G_4 = -29.845 \pm 0.007$ kcal/mol
$\Delta G_2 = -16.93 \pm 0.02$ kcal/2 mol

moglobin concentration, $[P_t]$, the median oxygen concentration $[\bar{X}]$ is calculated by direct integration of the binding curve [25]:

$$\int_{[O]}^{[\bar{X}]} \bar{Y}\, d \ln [X] = \int_{[\bar{X}]}^{\infty} (1 - \bar{Y})\, d \ln [X] \qquad (1)$$

Thus, $[\bar{X}]$ is the position on the concentration scale of a vertical bisector of the binding curve such that equal areas are obtained under the curve (to the left of $[\bar{X}]$) and above the curve (to the right). It should be noted that this simple determination of the median solely from the areas below and above the binding curve does not rely upon the curve's detailed shape, such as occurs in the numerical fitting for stepwise parameters. It thus depends only on 'first-order' effects, and is not subject to the potential pitfalls of shape analysis. Table 5 shows an illustrative set of medians obtained for 14 binding curves as a function of hemoglobin concentration at pH 8.5. The precision of the median, $[\bar{X}]$, calculated via eq. 1 from each binding curve, is seen to be approx. 1% of its value.

(5) The values of ${}^0K_2$, ${}^4K_2$, and $[\bar{X}]$, determined at a particular hemoglobin concentration $[P_t]$, are all that is needed to evaluate K_4 (the total tetramer affinity corresponding to the product of the four stepwise k'_{4i} values) and K_2 (the total dimer affinity over both binding steps). This is accomplished by using an extension [3] of the Wyman median relationship [25]:

$$K_4 = [\bar{X}]^{-4} \left\{ \frac{1 - {}^4f_2}{1 - {}^0f_2} \right\} \exp\left({}^0f_2 - {}^4f_2\right) \qquad (2)$$

where

$${}^4f_2 = \frac{\left(1 + 4{}^4K_2[P_t]\right)^{1/2} - 1}{2{}^4K_2[P_t]},$$

$${}^0f_2 = \frac{\left(1 + 4{}^0K_2[P_t]\right)^{1/2} - 1}{2{}^0K_2[P_t]}$$

Note that in the absence of dimeric species (${}^0f_2$ and ${}^4f_2$ both zero), eq. 2 reduces to the classical

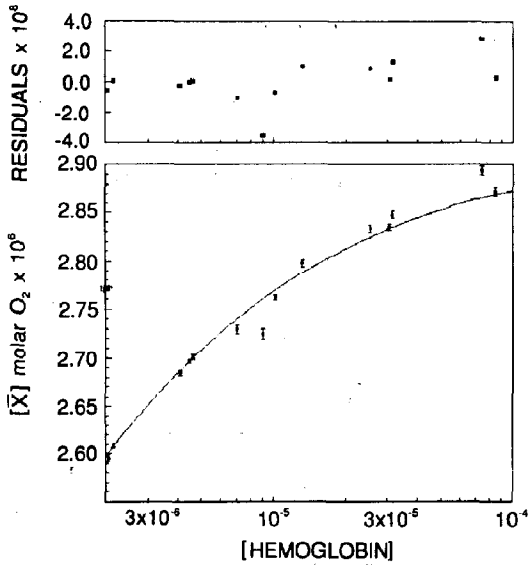


Fig. 2. Median oxygen concentrations [X] from 14 binding curves at different hemoglobin concentration. The solid curve represents the best fit to eq. 2. Conditions: 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM Na_2EDTA, pH 8.5, 21.5°C.

Table 6

Linkage constants for human hemoglobin resolved at pH 7.4 and 21.5 °C

Data set	$^0\Delta G_2$ (kcal)	$^4\Delta G_2$ (kcal)	ΔG_4 (kcal/$4O_2$)	ΔG_2 (kcal/$2O_2$)	$\Delta G_2/2$ (kcal/O_2)	Reference
1	−14.44±0.2	−8.05±0.1	−27.28±0.10	−16.82±0.03	−8.41±0.04	2
2	−14.23±0.2	−8.00±0.1	−27.15±0.08	−16.69±0.02	−8.34±0.03	7
3	−14.23±0.2	−8.00±0.1	−27.03±0.10	−16.62±0.03	−8.31±0.04	7
4	−14.23±0.2	−8.00±0.1	−27.06±0.09	−16.64±0.03	−8.32±0.04	7
5	−14.23±0.2	−8.00±0.1	−26.96±0.11	−16.64±0.04	−8.32±0.05	8
Mean values			−27.1 ±0.22	−16.68±0.02	−8.34±0.09	

Wyman relationship:

$$K_4 = [\bar{X}]^{-4} \tag{3}$$

which permits calculation of the free energy ($\Delta G_4 = 4RT \ln[\bar{X}]$) for complete tetramer oxygenation solely from the median position of the tetrameric isotherm [25]. Converting the equilibrium constants into corresponding free energies $^0\Delta G_2$, $^4\Delta G_2$, and ΔG_4, the free energy ΔG_2 for binding two oxygens onto the dimer is determined by conservation around the thermodynamic cycle:

$$\Delta G_2 = \left(\Delta G_4 + {}^0\Delta G_2 - {}^4\Delta G_2\right)/2 \tag{4}$$

While a single median at any known $[P_t]$ will in principle suffice to determine ΔG_4 and ΔG_2, it is desirable in practice to determine a series of $[\bar{X}]$ values at different protein concentrations as shown in fig. 2. This set of medians plus the corresponding values of $[P_t]$ is analyzed by nonlinear least-squares fitting according to eqs. 2 and 4 to obtain the best value of K_4 and K_2 (fig. 2). The precision with which ΔG_4 and ΔG_2 can be determined is extremely high, as illustrated in tables 5 and 6. For the binding curves of table 5, each median has a statistical precision of approx. 1%, leading to a precision of 0.02 kcal in the free energy ΔG_2 of dimer binding. Table 6 shows the overall reproducibility of these determinations at pH 7.4 and 21.5 °C from different studies and hemoglobin preparations.

(6) Finally, detailed shapes of the oxygen-binding curves are analyzed under constraints of the independently determined parameters just discussed. These four parameters provide three constraints (say $^0K_2$, K_4 and K_2) since any one of them is fixed by the other three. An especially useful set of remaining parameters then, is: $^0K_2/^1K_2$, $^3K_2/^4K_2$, $\sqrt{k'_{43}}$, and K_{coop2} (which is defined as $k'_{21}/\sqrt{K_2}$). It is, of course, possible to fit directly to other choices of parameters. Once a set of parameter values has been found (or even merely postulated) one can test them for goodness of fit to the data (i.e., the variance and distribution of residuals). We have used this procedure in section 3.2 to test the constants that have been recently proposed by Gibson and Edelstein [15] to explain the binding data of Mills and Ackers [7,9]. From any appropriately chosen set of seven parameters, all constants of the linkage scheme (fig. 1) are readily specified.

Note that $^3K_2/^4K_2$ is a direct reflection of quaternary enhancement and that the value of K_{coop2} directly reflects dimer cooperativity, since K_2 is fixed. Values of K_{coop2} less than unity, equal to unity, or greater than unity reflect positive cooperativity, noncooperativity, or negative cooperativity, respectively. When additional independent information exists, further constraints may be introduced so that the fitting problem requires fewer than these four parameters. A constraint that we have usually incorporated into these analyses, derived in part from the kinetic studies of Gibson and associates [26] as well as the analyses of Mills et al. [2], is that of noncooperativity in the dissociated dimers (see also ref. 27). Thus, when K_{coop2} is fixed at unity, the binding curves at various $[P_t]$ values are analyzed simultaneously for the remaining three parameters,

Table 7

Effects of dimer cooperativity and quaternary enhancement on resolved stepwise binding energies at pH 7.4 and 21.5 °C

Data set	Dimer cooperativity (kcal)	Quaternary enhancement (kcal)	Stepwise binding energies (kcal)					
			$\Delta G'_{41}$	$\Delta G'_{42}$	$\Delta G'_{43}$	$\Delta G'_{44}$	$\Delta G'_{22}$	$\sqrt{\text{Variance}}$
1	0.00	0.27	−5.47	−5.29	−7.82	−8.68	−8.41	0.005
	0.46	0.00	−5.50	−5.17	−7.97	−8.64	−8.64	0.005
	0.60	−0.07	−5.50	−5.19	−7.95	−8.63	−8.70	0.005
2	0.00	0.77	−5.37	−5.71	−6.96	−9.11	−8.34	0.004
	0.60	0.24	−5.42	−5.53	−7.31	−8.88	−8.64	0.005
	0.94	0.00	−5.45	−5.44	−7.45	−8.81	−8.81	0.005
3	0.00	0.86	−5.36	−5.79	−6.69	−9.12	−8.31	0.011
	0.60	0.36	−5.40	−5.64	−7.01	−8.97	−8.61	0.011
	1.10	0.00	−5.43	−5.52	−7.21	−8.87	−8.87	0.011
4	0.00	1.33	−5.43	−5.64	−6.34	−9.65	−8.32	0.005
	0.60	0.64	−5.47	−5.51	−6.81	−9.26	−8.62	0.005
	1.41	0.00	−5.50	−5.33	−7.19	−9.04	−9.03	0.005
5	0.00	0.74	−5.53	−5.16	−7.21	−9.06	−8.32	0.003
	0.60	0.38	−5.57	−5.18	−7.21	−9.00	−8.62	0.004
	1.10	0.00	−5.56	−5.31	−7.21	−8.88	−8.88	0.003

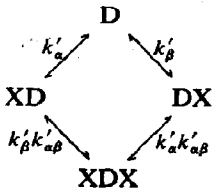
$^0K_2/^1K_2$, $^3K_2/^4K_2$, and $\sqrt{k'_{43}}$ by nonlinear least squares [3–6]. Representative values of oxygen-binding constants obtained by this procedure are shown in tables 7–9 for the same data sets of table 6. The first row of each data set gives the previously published values obtained under the constraint of dimer noncooperativity ($K_{coop2} = 1$), while the remaining values pertain to other fixed values of K_{coop2} (see section 3). It was found by Mills and Ackers [7] that almost equally good fits were obtained with the data sets of tables 7 and 9 by assuming the dimers to be cooperative (i.e., $K_{coop2} < 1$). In those cases compensating alterations to quaternary enhancement were also found since:

$$\left(^3K_2/^4K_2\right) \cdot K_{coop2} = \frac{\sqrt{K_2}}{k'_{44}} \tag{5}$$

This issue of compensation between quaternary enhancement and dimer cooperativity has been explored more extensively in the present study in relation to the constants reported by Gibson and Edelstein [15] and by Philo and Lary [16]. Since the values of k'_{22} and k'_{22} reported by Philo and Lary [16] require the dimers to be cooperative (as shown in section 2.2) we have explored the effects of imposing these values on the curve-fitting program to assess the consequent alteration of quaternary enhancement.

2.2. The affintiy and cooperativity of dimers

Combination of the kinetically derived value for k'_{22} (i.e., for binding the second oxygen onto dimers) with the equilibrium determination of K_2 (see tables 3 and 6) leads directly to the requirement for dimer cooperativity since $\sqrt{K_2} \neq K'_{22}$. Since Philo and Lary obtained separate apparent values for affinities of α- and β-subunits which they assigned solely to the second binding step, their values permit a complete specification of terms in the following dimer oxygenation scheme:



(6)

in which k'_α and k'_β are the respective intrinsic site

binding constants to α- and β-subunits of the dimer, and $k'_{\alpha\beta}$ is the cooperativity parameter. $k'_{\alpha\beta}$ defines the alteration in a site's affinity for oxygen when the other site is already occupied. The stoichiometric stepwise binding constants, corrected for statistical factors then are:

$$k'_{21} = 1/2(k'_\alpha + k'_\beta) \tag{7}$$

$$k'_{22} = \frac{2k'_\alpha k'_\beta k'_{\alpha\beta}}{k'_\alpha + k'_\beta} \tag{8}$$

The experimental subunit free energies in table 3 ($\Delta G_\alpha^{exp} = -8.82$ kcal and $\Delta G_\beta^{exp} = -8.49$ kcal) then correspond to the following equilibrium constants:

$$k_\alpha^{exp} = k'_\alpha k'_{\alpha\beta} = 3.53 \times 10^6\ M^{-1} \tag{9}$$

$$k_\beta^{exp} = k'_\beta k'_{\alpha\beta} = 2.01 \times 10^6\ M^{-1} \tag{10}$$

These relationships may be combined with results of the oxygenation curve median determination which provide a value of $2.35 \times 10^{12}\ M^{-2}$ for the equilibrium constant K_2 (corresponding to $\Delta G_2 = -16.68$ kcal of table 6).

$$K_2 = k'_\alpha k'_\beta k'_{\alpha\beta} = 2.35 \times 10^{12}\ M^{-2} \tag{11}$$

Then combining eqs 9 and 11 we can solve for all three parameters: $k'_{\alpha\beta} = 3.02$, $k'_\alpha = 1.17 \times 10^6$, $k'_\beta = 6.66 \times 10^5$. Hence $k'_{21} = 9.18 \times 10^5$, $k'_{22} = 2.56 \times 10^6$ and $K_{coop2} = 0.60$. We thus find that in order for the kinetic and equilibrium results to be compatible, the dissociated dimers must exhibit positive cooperativity in their two steps of oxygen binding. The magnitude of dimer cooperativity is determined at pH 7.4 and 21.5°C by the value of $(\Delta G'_{22} - \Delta G'_{21}) = -0.60 \pm 0.14$ kcal. Thus the affinity for the second oxygen bound would have to be 2.8-times that of the first. We have explored the effect of this apparent dimer cooperativity on analysis of the oxygen binding curves (section 3.1).

2.3. Affinity of tetramers at the last step of oxygenation

A much simpler analysis translates the kinetically derived tetramer affinities for α-subunits ($\Delta G'_\alpha = -8.75$ kcal) and β-subunits ($\Delta G'_\alpha = 8.68$ kcal) into the stoichiometric equilibrium constant for the fourth binding step:

$$k'_{44} = \frac{2k_\alpha^{exp} k_\beta^{exp}}{\left(k_\alpha^{exp} + k_\beta^{exp}\right)} = 2.94 \times 10^5 \tag{12}$$

so that $\Delta G'_{44} = -8.72 \pm 0.1$ kcal, in good agreement with the value of -8.62 ± 0.1 reported by Gibson and Edelstein [15] (see table 3).

3. Results and discussion

3.1. Analysis of the assembly-linked oxygenation data under constraints of the kinetically derived binding constants

In section 2.1, we described a procedure whereby information from kinetic and equilibrium techniques was combined to obtain an accurate determination of dimer oxygen affinity ΔG_2 under the same conditions as those used by Philo and Lary [16] and by Gibson and Edelstein [15] (see table 6). Moreover, this method does not depend on numerical analysis of the binding curve shapes. The resulting value for the free energy of totally oxygenating dimers, in combination with the kinetically derived constant assigned by Philo and Lary to the second step of dimer binding, leads to the requirement that dimers must have 0.6 kcal of cooperativity. We thus fixed the dimer free energies by this constraint and analyzed the oxygen-binding curves for remaining parameters of the linkage system (i.e., fitting to three parameters).

The analysis was first carried out with the five data sets at pH 7.4, 21.5°C, comprising 59 binding curves, along with the corresponding equilibrium and kinetic data for the dimer-tetramer association constants of unligated and fully ligated molecules. A primary goal was to test whether the oxygen-binding data could be fitted to a set of parameters of the linkage system that satisfied the values of k'_{22} and k'_{44} determined kinetically [15,16]. The results are shown in table 7. We found that in every data set except set 1 the oxygen-binding curves could not be fitted satisfactorily unless the value of k'_{44} was significantly higher than that estimated by the kinetic method. In data set 1 a

Table 8

Effects of dimer cooperativity and quaternary enhancement on resolved stepwise binding energies as a function of pH

pH (average values)	Dimer cooperativity	Quaternary enhancement	Stepwise binding energies (kcal)					
			$\Delta G'_{41}$	$\Delta G'_{42}$	$\Delta G'_{43}$	$\Delta G'_{44}$	$\Delta G'_{22}$	$\sqrt{\text{Variance}}$
7.4	0.0	0.79	−5.43	−5.52	−7.00	−9.13	−8.34	0.006
	0.60	0.32	−5.47	−5.40	−7.28	−8.95	−8.63	0.006
	0.91	0.0	−5.49	−5.35	−7.41	−8.85	−8.85	0.006
8.0	0.0	0.82	−5.86	−5.00	−8.42	−9.21	−8.39	0.004
	1.37	0.0	−5.90	(+5.79)	−(19.3)	−9.07	−9.07	0.004
8.5	0.0	0.97	−6.02	−6.85	−7.54	−9.43	−8.46	0.007
	1.37	0.0	−6.15	−6.49	−8.05	−9.15	−9.15	0.007
8.95	0.0	0.99	−5.99	−6.74	−7.81	−9.26	−8.27	0.008
	1.41	−0.02	−6.17	−6.06	−8.61	−8.96	−8.97	0.007
9.5	0.0	0.86	−5.98	−6.24	−8.78	−9.18	−8.32	0.004
	1.07	0.0	−6.22	(+5.50)	(−20.56)	−8.89	−8.87	0.005

value of 0.46 kcal in dimer cooperativity was sufficient to compensate for quaternary enhancement. This particular data set, included here for completeness, was previously found to be atypical [7–9]. In all other data sets the kinetically derived dimer values [16] can be accommodated in these fits only if the system exhibits both quaternary enhancement and dimer cooperativity, in which case the value required of k'_{44} is incompatible with that reported by Gibson and Edelstein [15], and by Philo and Lary [16] (see table 3). These oxygen-binding isotherms were also fitted under the constraint of no quaternary enhancement ($k'_{44} = k'_{22}$) as shown in table 7, by increasing the dimer cooperativity. However, dimer cooperativity could be made to compensate for quaternary enhancement only if both k'_{44} and k'_{22} disagree with the kinetically estimated values, and simultaneously the dimer cooperativity becomes much greater than allowed by the kinetically determined values of Philo and Lary [16]. The square root of the variance for these fits was 1% or less in all cases (table 7).

We also extended these analyses to a wider set of conditions including the data sets covering the temperature range 10–37°C [9], and the data sets

Table 9

Effect of dimer cooperativity and quaternary enhancement on resolved stepwise binding energies as a function of temperature

T (°C)	Dimer cooperativity	Quaternary enhancement	Stepwise binding energies (kcal)					
			$\Delta G'_{41}$	$\Delta G'_{42}$	$\Delta G'_{43}$	$\Delta G'_{44}$	$\Delta G'_{22}$	$\sqrt{\text{Variance}}$
10	0.0	1.11	−5.56	−5.82	−6.17	−9.74	−8.64	0.008
	1.35	0.0	−5.59	−5.66	−6.74	−9.30	−9.31	0.008
15	0.0	0.75	−5.60	−5.24	−7.22	−9.26	−8.50	0.004
	1.05	−0.08	−5.65	(+3.41)	(−16.62)	−8.95	−9.03	0.004
33	0.0	0.99	−5.52	−5.22	−7.27	−9.04	−8.06	0.002
	0.84	0.03	−5.64	(−6.89)	(−12.20)	−8.52	−8.48	0.002
37	0.0	0.87	−5.44	−5.15	−7.25	−8.71	−7.85	0.004
	1.13	0.02	−5.53	(−7.50)	(−20.11)	−8.43	−8.41	0.004

covering the pH range 7.4–9.5 [8]. Our goal was to test whether these data could be fitted to any sets of linkage parameters in which k'_{22} and k'_{44} are equal so that quaternary enhancement is nonexistent. The results are shown in tables 8 and 9. Extremely high dimer cooperativities were invariably required to make the fitted values of k'_{44} and k'_{22} equal, and this leads to physically unreasonable values of the remaining tetramer binding constants in five of the eight data sets (indicated by parentheses around the free energy values). Values are also listed for the noncooperative case and the case corresponding to 0.6 kcal of cooperativity for comparison. From the results of tables 7–9, we conclude that, over a wide range of conditions, the quaternary enhancement effect cannot be made to 'go away' through altered values of the remaining constants of the linkage system.

3.2. *Is quaternary enhancement an artifact of data analysis procedures?*

Mills and Ackers [9] presented a set of average values (see table 10, column A) for three data sets previously analyzed [7]. It was found that the value for $\Delta G'_{44}$ of -9.31 ± 0.37 kcal, compared with $\Delta G'_{22}$ of -8.33 ± 0.2 kcal, indicated an average quaternary enhancement of 0.98 kcal. These results were questioned by Gibson and Edelstein [15] with the proposal that the original oxygen-binding curves might be fitted just as well to the values shown in column B of table 10 (see table I of ref. 15 along with their discussion of the linkage square on p. 518). Gibson and Edelstein based their analysis on an attempt to 'regenerate the original data' in terms of a single tetrameric binding curve from just four of the Mills and Ackers constants (i.e., the four $\Delta G'_{4i}$ of table 10, column A). They arrived at the values in column B, by fixing $\Delta G'_{44}$ at -8.62 kcal, $^4\Delta G_2$ at -8.00 kcal, and $\Delta G'_{22}$ at -8.32 kcal, so that $^3\Delta G_2$ is -7.70 kcal by conservation around the cycle, followed by subsequent adjustment in the values of $\Delta G'_{43}$ and $\Delta G'_{41}$. A criterion they used for choosing these remaining values was that a tetrameric curve calculated from them was judged to be reasonably close to the regenerated data.

We have tested the proposal of Gibson and Edelstein that the values of column B should provide an equally good fit to the original data of Mills and Ackers [7,9] as those of column A so that "on the basis of the new values the hypothesis of quaternary enhancement is unnecessary" (ref. 15, p. 519). We carried out the nonlinear least-squares fits [3,5] to each of the original three sets of binding curves (these data sets happen to be the same as sets 3–5 of tables 6 and 7 in the present paper). The criteria for goodness of fit included

Table 10

Binding curve analysis using constraints proposed by Gibson and Edelstein

Gibbs free energy	Found by Mills and Ackers [9] (A)	Proposed by Gibson and Edelstein [15] [a] (B)	This study	
			Floating $^4\Delta G_2$ (C)	Floating K_{coop2} (D)
$^0\Delta G_2$	-14.23 ± 0.1	-14.35 ± 0.1	(fixed at column B value)	
$^3\Delta G_2$	-7.02 ± 0.33	-7.70 ± 0.33	-6.99 ± 0.04	-6.71 ± 0.08
$^4\Delta G_2$	-8.00 ± 0.1	-8.00 ± 0.1	-7.11 ± 0.03	(fixed at column B value)
$\Delta G'_{21}$	-8.33 ± 0.1	-8.32 ± 0.1	(fixed at column B value)	-9.22 ± 0.05
$\Delta G'_{22}$	-8.33 ± 0.1	-8.32 ± 0.1	(fixed at column B value)	-7.33 ± 0.06
$\Delta G'_{41}$	-5.39 ± 0.19	-5.05 ± 0.1	(fixed at column B value)	
$\Delta G'_{42}$	-5.71 ± 0.94	-5.71 ± 0.1	(fixed at column B value)	
$\Delta G'_{43}$	-6.67 ± 0.53	-7.38 ± 0.1	(fixed at column B value)	
$\Delta G'_{44}$	-9.31 ± 0.37	-8.62 ± 0.1	(fixed at column B value)	
Average variance of fits	6.0×10^{-5}	8.7×10^{-3}	8.8×10^{-4}	4.0×10^{-3}

[a] See table I and linkage square on p. 518 of ref. 15.

the usual requirements of convergence to a minimum in the variance, randomness in the distribution of residuals, and narrowness in the confidence intervals associated with the parameter values [2,3].

It should be noted that column B prescribes fixed values for all parameters of the linkage system so that additional parameter estimation is precluded without relaxation of some values. It is nevertheless a simple matter to test for goodness of fit of the Gibson and Edelstein parameters to the original data. This analysis gave variances of 6.9×10^{-3}, 3.1×10^{-3}, and 3.1×10^{-3} for data sets 2–4 respectively. (The value of 8.7×10^{-3} listed in table 10 is the mean variance.) These values are two orders of magnitude higher than those of the corresponding fits of the same data to the parameters of column A, where the mean variance for the three data sets was 6.0×10^{-5}. Highly skewed distributions of residuals (not shown) were exhibited by fits to the column B values, as compared with those of column A. It is clear from these results that the original data cannot be fitted nearly as well to the parameter values of column B which allow only 0.3 kcal quaternary enhancement as compared to the values of column A which allow approx. 1 kcal.

It was of interest to explore whether the four tetrameric binding constants proposed by Edelstein and Gibson could nevertheless conform to the oxygenation data by allowing adjustment of other constants. We, therefore, carried out fits in which the six parameters were fixed at the column B values (i.e., $\Delta G'_{4i}$ $(i = 1\text{–}4)$, ${}^{0}\Delta G_2$, and K_{coop2}) and ${}^{4}\Delta G_2$ was allowed to float, leading to altered values of ${}^{3}\Delta G_2$ as well. The results are shown in column C of table 10. The variance is approx. 10-fold poorer than that of column A, and the best value of ${}^{4}\Delta G_2$ is -7.1 kcal, which is far outside the acceptable range for this parameter (table 4). Thus, the results of column D must be rejected on the basis of the independent determinations of ${}^{4}\Delta G_2$ which yield a value of -8.00 ± 0.1 kcal. This provides a striking example of the value of additional information that is fed into the analysis when oxygen-binding parameters are constrained by subunit assembly values.

A further test of interest in light of the dimer cooperativity issues discussed in section 3.1 was to float K_{coop2} while fixing ${}^{4}\Delta G_2$ at the independently determined value of -8.00 ± 0.1 kcal and also fixing the four stepwise tetramer constants $\Delta G'_{4i}$. Then, as K_{coop2} floats, so do the dimer constants k'_{21} and k'_{22} as well as ${}^{3}\Delta G_2$. Results of these analyses are given in column D of table 10. Quaternary enhancement is 1.29 kcal while dimers are anticooperative by 1.89 kcal! Floating both K_{coop2} and ${}^{4}\Delta G_2$ (not shown) did not improve this situation. The results of column D can be rejected on the grounds that the goodness of fit is 50-fold worse than that of column A, among other criteria.

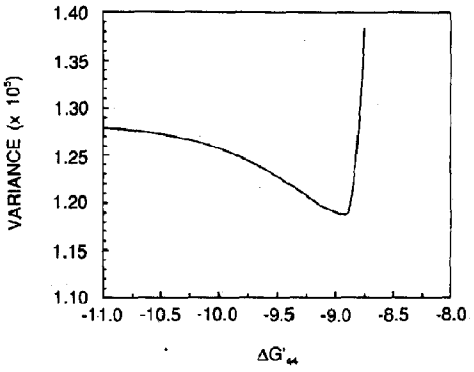


Fig. 3. Variance of fit in least-squares analysis of data set 5 (table 7) as a function of fixed values of the last tetramer binding constant $\Delta G'_{44}$. Floating parameters in these fits are $\Delta G'_{41}$, $\Delta G'_{42}$ and $\Delta G'_{43}$.

Fig. 3 illustrates the severe limitations on values of $\Delta G'_{44}$ that provide a given goodness of fit below and above the best (minimum) value. It is seen that forcing $\Delta G'_{44}$ to lower values that would approach those of the kinetically determined constants has a drastic effect on the ability of the remaining parameters to accommodate the data.

3.3. Relationship of quaternary enhancement to the two-state MWC model

Gibson and Edelstein [15] have advanced an argument (attributed by them to "Ackers and co-workers") that the existence of quaternary enhancement would disprove the two-state MWC model of allosteric regulation. In fact, Ackers and

Johnson [30] had explicitly demonstrated the exact opposite: the subunit assembly-linked oxygenation data (e.g., data set 1, table 7 of the present study) was found to provide an excellent fit to a model in which the tetramers conform strictly to the two-state MWC model while the oxygen affinity of dissociated dimers is lower than that of R-state tetramers. The dimers of this model thus comprise a 'third affinity state' so that the entire linkage system becomes a 'three-state system'. While quaternary enhancement requires three affinity states for the entire system, only two of those states necessarily pertain to tetramers and those two were found to conform to the MWC two-state rules. Conformity to this model was also demonstrated for all of the data sets of table 9 over a range of temperatures. The subunit assembly-linked oxygenation data thus do not rule out the two-state MWC model [33] which makes no assumptions about the properties of dissociated protomers. The data do, however, exhibit incompatibility with the 'polysteric model' of Colosimo and Wyman [34], which extends the MWC model to include protomer dissociation. Of course, the presence of dimer cooperativity as required by the Philo and Lary constants [16] would also require, at the minimum, a three-state model for the entire system.

While the coupling of binding equilibria involving dissociated dimers to the four stepwise tetramer binding reactions can provide a powerful methodological tool (as illustrated in section 3.2), there is no necessary relationship between the stepwise binding constants of the two kinds of molecules; a very large number of mechanisms is formally possible within the general energy conservation requirements. To say otherwise would be a violation of thermodynamic principles. It is also worth noting, however, that the data analyzed by Ackers and Johnson [30] were found to fit equally well to a three-state model in which the tetramers have three affinity states and the dimers have an affinity identical to that of one of the three tetramer states. While the existence of quaternary enhancement strongly suggests the operation of 'oppositely directed constraints' within tetramers that are responsive to ligation, other interpretations must also be allowed [7].

3.4. Possible sources of incompatibility between different techniques

In this study, we have analyzed the problem of incorporating the new kinetically derived binding constants along with the other independently determined parameters into the analysis of oxygen-binding data for resolving the linkage system of fig. 1. We found that parameter sets which are compatible with the kinetically derived binding constant for dimers [16] clearly require quaternary enhancement at the last step of tetramer binding, while simultaneously requiring dimer cooperativity. While dimer cooperativity and quaternary enhancement compensate reciprocally, the parameter sets that have sufficient dimer cooperativity to eliminate quaternary enhancement require both dimeric and tetrameric binding constants that disagree with the kinetically derived values.

We cannot presently identify the origins of the discrepancy between results of the recent kinetic measurements for oxygen binding and those of the other four techniques which, together, provide self-consistent sets of linkage parameters over a wide range of conditions. Although it is possible that these four independent techniques provide a self-consistent, yet biased, picture of the linkage system's energetics, such skewing would certainly be small relative to the extremely conservative error limits employed (e.g., table 10) and the multiple independent controls used to validate the $^4K_2$ and $^0K_2$ values. Different extinction coefficients for the intermediate species of, say 25%, would lead to roughly comparable errors in the binding constants, or errors of less than 0.2 kcal in the resolved free energies. The restrictions imposed on the four stepwise tetramer constants by constraining the oxygen-binding data against the values of $^0K_2$, $^4K_2$, K_2 and K_4 are very stringent, as illustrated by the results of table 10. While the stringency of these constraints increases with the fraction of dimeric species actually present throughout the measured binding curves (especially in regard to the stepwise dimer constants) the major constraints are operative for binding curves reflecting essentially only tetramers. By attempting to collapse the multidimensional linkage data base into a single tetrameric binding curve,

Gibson and Edelstein [16] may have lost critical information in their fitting exercise. While we agree with their point of view regarding the long-standing difficulties of obtaining unique stepwise constants from a single binding curve, and it was this realization that led us to the multidimensional strategy [1–3], it seems that to eliminate the additional information yielded by this approach would be a step backward.

Several aspects of the new kinetic results are troubling. Assignment of the rate processes for oxygen dissociation and rebinding is dependent on the assumption that triply ligated and fully ligated tetramers can each exist in only two conformational states (R or T). This premise was used to exclude possible conformational changes from other states as contributing to the observed rate effects [15,16]. Such a strategy for assignment of the kinetic processes would seem questionable in view of current evidence for more than two allosteric states [6] and of careful studies indicating a different structure for the triply ligated tetramers with oxygen binding. For example, Makino and Sugita [29] found the rate of sulfhydryl titration for the triply ligated species to be faster than for either oxyhemoglobin or deoxyhemoglobin, while rates of the latter two species are traditional markers of 'R-state' and 'T-state' molecules.

A second problem is the absence of amplitude analysis that would provide credence to the rate information. In the oxygen rebinding experiments of Philo and Lary [16] the assignment of rates to dimeric and tetrameric species was made by setting the hemoglobin concentrations to low and high values so that the proportion of dimers to tetramers changed. The apparent rates were found to be insensitive to hemoglobin concentration, precluding an assessment of whether the relative amplitudes change with concentration according to the same law which governs an association-dissociation equilibrium. Because of this insensitivity, the standard controls for validating equilibrium constant determinations by rate information are sorely needed (i.e., to test whether the mass-action parts of the process are solely being reflected in the observed rates). An earlier study by Philo et al. [32] using the same techniques demonstrated time-dependent complexities for the dissociated chains (attributed by them to multiple conformational forms and inverted hemes) and failed to produce equilibrium binding constants. Basic controls would go further toward establishing validity of these methods than does the observation of high precision in fitting the data to a sum of two exponentials [16].

A surprising aspect of the kinetic results of Philo and Lary [16] is that they require the dissociated dimers to be significantly cooperative. While small amounts of dimer cooperativity or anticooperativity have not been rigorously excluded by earlier studies, a wealth of information, both kinetic and equilibrium, has indicated that dimer cooperativity must be quite small, or nonexistent [2,26,27]. The new kinetically derived equilibrium constants require dimer cooperativities of 0.6 kcal (or as much as 1.4 kcal in order to eliminate quaternary enhancement; tables 7–9). By contrast, the average cooperativity in tetramers per binding step would be only 0.89 kcal, even using the values of Gibson and Edelstein (table 10). The finding, illustrated by table 2, that the sum of the free energies for α_2 and β_4 binding is identical to that determined for dimers by the medians of concentration-dependent hemoglobin-binding curves argues strongly against significant dimer cooperativity. If the dimers exhibited such cooperativity, the almost exactly compensating affinity increase (for the second step) and decrease (for the first step) would be a remarkable 'coincidence', especially over a range of changing solution conditions.

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