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# Consequences of neglecting the hemoglobin concentration on the determination of Adair binding constants

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It is commonly believed that the tetrameric Adair constants for oxygen binding to human hemoglobin can be evaluated from a single oxygenation experiment at 'high' hemoglobin concentration without considering the consequence of the presence of $\alpha\beta$ dimers. We present examples which demonstrate that this is a very dangerous assumption. Without a knowledge of the complete oxygenation-linked dimer-tetramer association reaction ($\alpha\beta X_i \leftrightarrow (\alpha\beta)_2 X_j$), it is impossible to predict a priori how high of a hemoglobin concentration would be required to make this assumption. Furthermore, without a knowledge of the complete oxygenation-linked dimer-tetramer association reaction, it is impossible to predict a priori the direction and magnitude of the systematic errors which are induced by making this assumption.

## 1. Introduction

Hemoglobin is probably the most widely studied of all proteins which show cooperativity. Hemoglobin has been used extensively for mechanistic and model studies of cooperativity (e.g., see refs 1–9) because of the large amount of experimental data which is available. As a consequence of these mechanistic and model studies, even more experimental data has become available.

In a number of these studies [1–9], it has become obvious that the ability to distinguish between mechanistic models is dependent upon the evaluation of highly accurate Adair binding constants. The level of discrimination required in current work requires that the free energy changes for the successive oxygen-binding steps be evaluated to within a few tenths of a kcal/mol [7–9]. The determination of accurate Adair binding constants is dependent on precise experimental data and its correct interpretation. The correct interpretation of the experimental data requires that all of the assumptions of the method of data analysis be met.

The most commonly used method of analysis for oxygen-binding data is nonlinear least-squares. Nonlinear least-squares analysis is a two-step process. First, the parameter values (Adair constants) with the highest probability of being correct are evaluated [10,11] and second the statistical confidence (standard error) of these parameters must be evaluated [7,10–12]. One of the assumptions which must be made in order to utilize nonlinear least-squares to evaluate parameter values is that the functional form of the equation which is used to describe the data is correct [10]. It is impossible to predict a priori how much systematic error will be introduced into a parameter value (Adair constant) by using an incorrect formulation [10].

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The theoretical [13] and experimental [14–18] demonstration of the linkage between oxygen binding and dimer-tetramer subunit assembly in human hemoglobin $A_0$ ($\alpha\beta X_i \leftrightarrow (\alpha\beta)_2 X_j$) is well documented. This work [13–18] has demonstrated that seven equilibrium constants are required to describe the oxygen-binding behavior of human hemoglobin if only the oxygen concentration is varied, i.e., in the absence of allosteric effectors. Of these seven equilibrium constants, two describe the oxygen-binding behavior of the $\alpha\beta$ dimer of hemoglobin, four describe the oxygen-binding behavior of the $(\alpha\beta)_2$ tetramer of hemoglobin and one describes the dimer-to-tetramer subunit assembly of the hemoglobin [13].

It is commonly believed that the reason the seven-parameter formulation was used [3–7,9–17], instead of the more convenient four-parameter Adair formulation [19,20], is that it was required because the oxygen-binding curves were measured at very low hemoglobin concentrations. In actuality, the experiments were designed such that the oxygen-binding curves were measured at very low hemoglobin concentration so that the protein-concentration-dependent linkage between subunit assembly and oxygenation binding would provide two dimensions (hemoglobin and oxygen concentration) to probe the mechanism of cooperativity in hemoglobin. It was correctly assumed that the measurement of oxygen binding as a function of both oxygen and hemoglobin concentration would provide a better resolution of the problem. This improved resolution is analogous to that which is obtained with two-dimensional electrophoresis or two-dimensional NMR.

It is also commonly believed that the tetrameric Adair constants can be evaluated from a single oxygenation experiment at 'high' hemoglobin concentration. In the early 1970's it was assumed that 60 $\mu$M heme was sufficient [21–25]. It was subsequently demonstrated [26] that this value was insufficient. It is currently assumed by some laboratories [27] that 600 $\mu$M heme is sufficiently high. Other laboratories have used somewhat higher values (1–2 mM heme), but still assume that they have used a hemoglobin concentration which is sufficient to remove the contribution of $\alpha\beta$ dimers [28].

There are two purposes of this work. First, to review the advantages of using the complete seven-parameter linkage between ligand binding and subunit assembly. Second, to demonstrate that it is impossible to assume a priori that the hemoglobin concentration is sufficient to allow the use of the four-parameter tetramer Adair formulation for the analysis of hemoglobin-oxygen binding data.

## 2. Methods

Hemoglobin $A_0$ is known to undergo a reversible dimer ($\alpha\beta$) to tetramer ($(\alpha\beta)_2$) association reaction which is linked to the degree of oxygenation of both the dimeric and tetrameric species [13]. These reactions are shown schematically in fig. 1. The stepwise Adair constants for dimer and tetramer and the dimer-to-tetramer assembly constants are defined by this scheme.

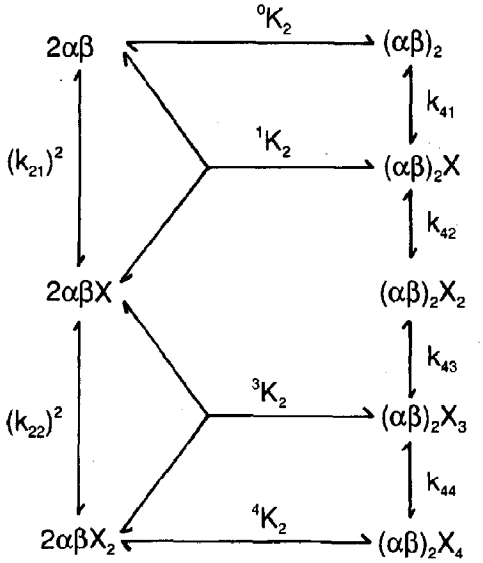


Fig. 1. The seven-parameter oxygenation-linked dimer, $\alpha\beta$, to tetramer, $(\alpha\beta)_2$, reaction scheme as defined by Ackers and Halvorson [13]. The $^iK_2$s are the subunit assembly constants to form a tetramer with $i$ ligands bound. The $k_{2i}$s and $k_{4i}$s denote the stepwise Adair constants for binding oxygen to hemoglobin dimers and tetramers, respectively.

The seven-parameter binding formulation is:

$$\bar{Y}_{2,4} = \left\{ Z_2' + \left[ Z_4' \left\{ \left( Z_2^2 + 4^0 K_2 Z_4 [\mathrm{P_t}] \right)^{1/2} - Z_2 \right\} \times (4Z_4)^{-1} \right] \right\} \times \left\{ Z_2 + \left( Z_2^2 + 4^0 K_2 Z_4 [\mathrm{P_t}] \right)^{1/2} \right\}^{-1} \quad (1)$$

where

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

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

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

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

where $K_{21}$ and $K_{22}$ are the product Adair constants for oxygen binding to the $\alpha\beta$ dimer, $K_{41}$, $K_{42}$, $K_{43}$ and $K_{44}$ the tetrameric product Adair constants, ${}^0K_2$ the dimer-tetramer association constant ($\alpha\beta \leftrightarrow (\alpha\beta)_2$) at zero oxygen concentration, [X] the oxygen concentration, and [Pt] the heme concentration. The product constants are defined in terms of the stepwise constants as:

$$K_{21} = k_{21} \quad (6)$$

$$K_{22} = k_{21}k_{22} \quad (7)$$

$$K_{41} = k_{41} \quad (8)$$

$$K_{42} = k_{41}k_{42} \quad (9)$$

$$K_{43} = k_{41}k_{42}k_{43} \quad (10)$$

$$K_{44} = k_{41}k_{42}k_{43}k_{44} \quad (11)$$

For a more complete description of these equations, the reader is referred to the paper of Ackers and Halvorson [13].

The four-parameter Adair formulation is:

$$Y_4 = Z_4'/4Z_4 \quad (12)$$

The 'induced systematic errors' are evaluated as:

$$\delta\Delta G_{4i} = RT \ln(K_{4i,\mathrm{app}}/K_{4i}) \quad (13)$$

$$\delta\Delta g_{4i} = RT \ln(k_{4i,\mathrm{app}}/k_{4i}) \quad (14)$$

where $k_{4i}$ and $K_{4i}$ denote the actual stepwise and product tetramer Adair constants and the subscript app refers to the values determined by the use of the four-parameter tetramer Adair formulation.

Simulated oxygenation curves were generated on the basis of the hemoglobin-concentration-dependent seven-parameter oxygen-binding formulation (eqs 1–5) [13], and were analyzed by a nonlinear least-squares procedure [10] in terms of the four-parameter tetrameric Adair formulation (eq. 12) [19,20]. By comparing the results with the initial values, we can directly evaluate the systematic errors (eqs 13 and 14) which were induced by the use of the incorrect fitting equation.

Each simulated data set had 50 data points which were equally spaced and encompassed a range of fractional saturation from 0.0 to 0.99. These data were simulated to approximate the data which are obtained with an Imai-type oxygenation apparatus [3–7,9,13–17,21–25]. However, the conclusions which we present are a function of the form of eqs 1–5 and 12, and consequently are common to all types of oxygenation instruments.

## 3. Results

The objective in this section is to evaluate how much systematic error is induced in the values of the tetramer Adair binding free energies by the use of the tetramer Adair formulation (eq. 12) at less than infinite concentration. Unfortunately, this can be solved for specific values of the linkage free energies, and distributions of oxygen and hemoglobin concentrations, but not for the general case. Furthermore, it is virtually impossible to evaluate the induced systematic errors at all possible values of the free energy changes due to the number of possible permutations.

Consequently, we decided to evaluate the induced systematic errors for two specific cases from the literature; hemoglobin $A_0$ [17] and hemoglobin Kansas [16]. The Kansas equilibrium constants [16] in table 1 were evaluated from a data set which contained nine separate oxygen titrations (332 data points) at hemoglobin concentra-

Table 1

Seven-parameter linkage constants

The values for hemoglobin A were taken from ref. 17; the experimental conditions were 0.1 M Tris, 0.1 M NaCl, 1.0 mM $Na_2EDTA$ (pH 7.4) at 21.5°C. The equilibrium constants for hemoglobin Kansas were taken from ref. 16; the experimental conditions were 0.05 M Tris, 0.1 M NaCl, 1.0 mM EDTA (pH 7.5) at 20°C.

| | Hb A | Hb Kansas |
|---|---|---|
| $K_{21}$ | $3.09\times10^{6}$ | $1.93\times10^{6}$ |
| $K_{22}$ | $2.39\times10^{12}$ | $6.00\times10^{11}$ |
| $K_{41}$ | $4.26\times10^{4}$ | $8.44\times10^{4}$ |
| $K_{42}$ | $8.22\times10^{8}$ | $1.50\times10^{9}$ |
| $K_{43}$ | $7.96\times10^{13}$ | $1.43\times10^{13}$ |
| $K_{44}$ | $1.24\times10^{20}$ | $2.07\times10^{17}$ |
| ${}^{0}K_2$ | $4.40\times10^{10}$ | $1.59\times10^{10}$ |

tions ranging from 6.24 mM heme down to 0.362 μM heme, a gel permeation determination of the dimer-to-tetramer association constant for the fully liganded hemoglobin (${}^{4}K_2$), and a kinetic determination of dimer-to-tetramer association constant for the unliganded hemoglobin (${}^{0}K_2$). The hemoglobin $A_0$ equilibrium constants presented by Chu et al. [17], and listed in table 1, are actually composite values for five pH 7.4 data sets; one published in ref. 17, one in ref. 14, and three complete data sets which were measured by Mills and Ackers [15]. Each of these hemoglobin $A_0$ data sets contains multiple oxygen titrations at different hemoglobin concentrations and independent determinations ${}^{0}K_2$ and ${}^{4}K_2$. The two sets of equilibrium constants (table 1) were measured under nearly identical solvent conditions, but represent two different hemoglobins, studied at two different laboratories. Furthermore, Atha et al. [16] used both the Imai cell and the Gill cell to perform the oxygen titrations, while Chu et al. [17] obtained data with the Imai cell.

In the first example, sets of synthetic data were generated at a series of different hemoglobin concentrations utilizing the seven equilibrium constants given in ref. 17 and using eqs 1–5. Each of these data sets was then analyzed by nonlinear least-squares [10] according to eq. 12 to obtain a set of 'apparent' Adair binding constants. We then compared the apparent free energy changes for each of the oxygenation steps of the tetramer

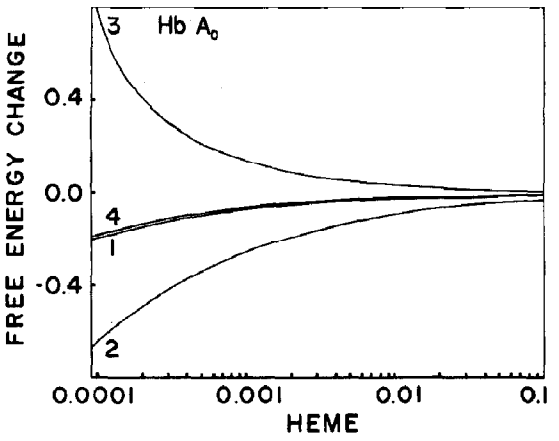


Fig. 2. The induced systematic error for hemoglobin $A_0$ [17]. Some of the values are listed in table 2. These were evaluated as in table 2 by the use of eq. 13.

at each of the hemoglobin concentrations with the value which was used to generate the data. The difference between these numbers is a direct measure of the systematic errors induced in tetramer Adair parameters by the use of the incorrect four-parameter Adair formulation (eq. 12). A plot of the differences of the free energy changes corresponding to $K_{41}$, $K_{42}$, $K_{43}$ and $K_{44}$ is shown in fig. 2. The values of the induced systematic errors at 1.0 and 3.0 mM are also listed in table 2. The largest induced systematic error at 1.0 mM heme

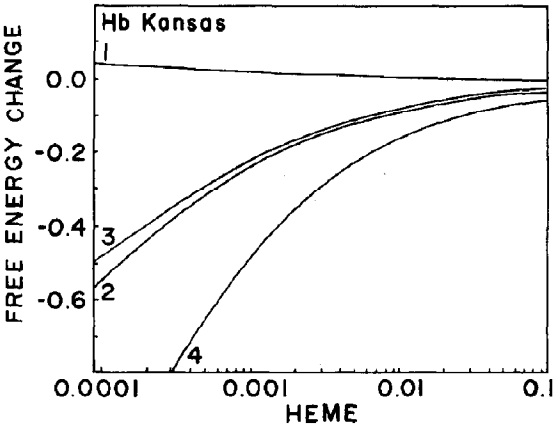


Fig. 3. The induced systematic error for hemoglobin Kansas [16]. Some of the values are presented in table 3. This was evaluated as in table 2 by the use of eq. 13.

Table 2

Induced systematic errors for hemoglobin $A_0$

The $\delta\Delta G_{4i}$s are the induced systematic errors in the free energy changes which correspond to the tetrameric product Adair constants. The $\delta\Delta g_{4i}$s are the induced systematic errors in the free energy changes which correspond to the tetrameric stepwise Adair constants. $\sigma^2$ denotes the variance which was induced by the use of the incorrect functional form for the nonlinear least-squares data analysis, i.e., eq. 12. The units of hemoglobin concentration are molar hemoglobin monomers (heme).

| | [Heme] | |
|---|---|---|
| | 1.0 mM | 3.0 mM |
| $\delta\Delta G_{41}$ | −0.07 | −0.04 |
| $\delta\Delta G_{42}$ | −0.26 | −0.16 |
| $\delta\Delta G_{43}$ | 0.14 | 0.07 |
| $\delta\Delta G_{44}$ | −0.06 | −0.03 |
| $\delta\Delta g_{42}$ | −0.19 | −0.12 |
| $\delta\Delta g_{43}$ | 0.40 | 0.23 |
| $\delta\Delta g_{44}$ | −0.20 | −0.10 |
| $\sigma^2$ | $1.11\times10^{-7}$ | $3.84\times10^{-8}$ |

is 0.40 kcal/mol and 0.23 kcal/mol at 3.0 mM heme.

In the second example we utilized the equilibrium constants for hemoglobin Kansas reported by Atha et al. [16]. The resulting plots of the induced systematic errors for hemoglobin Kansas are given in fig. 3 and the values at 1.0 and 3.0 mM heme are given in table 3. The largest systematic error is 0.48 kcal/mol at 1.0 mM heme and 0.29 kcal/mol at 3.0 mM heme.

Table 3

Induced systematic errors for hemoglobin Kansas

The $\delta\Delta G_{4i}$s are the induced systematic errors in the free energy changes which correspond to the tetrameric product Adair constants. The $\delta\Delta g_{4i}$s are the induced systematic errors in the free energy changes which correspond to the tetrameric stepwise Adair constants. $\sigma^2$ denotes the variance which was induced by the use of the incorrect functional form for the nonlinear least-squares data analysis, i.e., eq. 12. The units of hemoglobin concentration are molar hemoglobin monomers (heme).

| | [Heme] | |
|---|---|---|
| | 1.0 mM | 3.0 mM |
| $\delta\Delta G_{41}$ | 0.02 | 0.01 |
| $\delta\Delta G_{42}$ | −0.24 | −0.15 |
| $\delta\Delta G_{43}$ | −0.22 | −0.14 |
| $\delta\Delta G_{44}$ | −0.48 | −0.29 |
| $\delta\Delta g_{42}$ | 0.26 | 0.16 |
| $\delta\Delta g_{43}$ | −0.02 | −0.01 |
| $\delta\Delta g_{44}$ | 0.26 | 0.15 |
| $\sigma^2$ | $9.74\times10^{-8}$ | $3.96\times10^{-8}$ |

## 4. Discussion

Originally, the choice was made to utilize the subunit dissociation because it was felt that the hemoglobin concentration would provide a better resolution of the oxygen-binding properties of human hemoglobin $A_0$. In retrospect, it is clear that the approach which was taken by Ackers and co-workers [3–7,9,13–15,17,26] and others [16] has a number of advantages. These include:

(1) Better resolution.
(2) Ability to combine multiple types of experiments.
(3) More flexible choice of parameters.
(4) Inability to predict how 'high' a concentration is needed to neglect dimer formation.

The use of the full linkage scheme (eqs 1–5) clearly offers more resolution than the tetramer Adair formulation (eq. 12). It does involve more parameters and is more complex, however, it also allows the use of multiple binding curves at different hemoglobin concentrations as well as kinetic and equilibrium data which pertain to the subunit assembly reactions. For example, the Kansas equilibrium constants [16] in table 1 were evaluated from a data set which contained nine separate oxygen titrations at hemoglobin concentrations ranging from 6.24 mM heme down to 0.362 μM heme, a gel permeation determination of the dimer-to-tetramer association constant for the fully liganded hemoglobin, and a kinetic determination of dimer-to-tetramer association constant for the unliganded hemoglobin. The variation in the hemoglobin concentration adds an additional dimension of resolution to the study of hemoglobin cooperativity. This is analogous to the use of two-dimensional vs one-dimensional electrophoresis and/or the use of two-dimensional vs one-dimensional NMR.

An example of the use of the added resolvability provided by the use of the linkage scheme (fig.

1) is presented in the Ackers and Johnson [3] extension of the concerted (MWC) model [1] to include ligand-linked subunit assembly in human hemoglobin. In the simplest version of this extended model, the dimer is equivalent to half of an R-state tetramer. This simplest version of the extended model was found to be excluded unequivocally by data for both hemoglobins $A_0$ and Kansas when the $\alpha$ and $\beta$ chains have equal binding affinities. When this two-state model was modified to permit nonequivalent affinities for the chains, the model could describe the hemoglobin Kansas data but not that of hemoglobin $A_0$. A more complex model, in which the dimers are allowed to exist in a state different from either the R-state or T-state tetramers, was found to be consistent with the data for hemoglobin $A_0$. It would have been nearly impossible to make these observations about the model-dependent relationship of dimer and tetramer binding properties without having data which reflects the properties of both dimeric and tetrameric species and the interaction between them.

The original pH 7.4 data of Mills et al. [14], the three additional pH 7.4 data sets of Mills and Ackers [15], the five temperature-dependent data sets of Mills and Ackers [29], the six pH-dependent data sets of Chu et al. [17], and the hemoglobin Kansas data set of Atha et al. [16] all contain multiple types of experimental measurements in addition to oxygen-binding data using an Imai cell. All of these sets of data include an evaluation of the fully oxygenated dimer-to-tetramer association constant, ${}^4K_2$, measured by gel permeation column chromatography. All of the data sets include a kinetic determination of the dissociation and association rates for the unliganded dimer-to-tetramer reaction. The Kansas data combines oxygen-binding data from both an Imai cell and a Gill cell [16]. The combination, and consistency, of these different types of experiments add a significant amount of information to the data sets, more than sufficient to offset the additional parameters required by the linkage scheme (fig. 1).

When using the four-parameter Adair formulation the choice of parameters is restricted to either the $k_{4i}$s, the $K_{4i}$s, or their corresponding free energy changes. The reader will note that ten equilibrium constants are defined in the dimer-to-tetramer linkage scheme (fig. 1), but only seven of these are independent. This provides considerable flexibility in the choice of parameters to be estimated by the nonlinear least-squares procedure. Consequently, parameters can be chosen because they are of particular interest, or allow assumptions to be made and tested, or can be measured independently. For example, Mills et al. [14] chose to use ${}^0K_2$, ${}^4K_2$ and $K_{44}$ which then defines $K_{22}$ because of the cyclic nature of the scheme. Both ${}^0K_2$ and ${}^4K_2$ are measured independently. The dimeric species were assumed to be noncooperative which, because of the cyclic nature, defines $k_{21}$ and $k_{22}$. They chose the ratio of ${}^0K_2/{}^1K_2$ as a fitting parameter which defines $K_{41}$ (and $k_{41}$). They chose the ratio of ${}^3K_2/{}^4K_2$ as a fitting parameter which defines $k_{44}$. The choice of the ratio of ${}^3K_2/{}^4K_2$ is also of particular interest because it is directly proportional to the 'quaternary enhancement effect' [15]. If the ratio of ${}^3K_2/{}^4K_2$ is significantly different from 2 (a statistical factor) then the quaternary enhancement effect [15] exists. This effect is currently of great interest in the literature [7–9]. By making the quaternary enhancement effect a fitting parameter, the investigators were able to carry out direct evaluation of the parameter and the associated statistical confidence (standard error). This is again an example of how a data set which includes data pertaining to the dimeric species' properties can provide information which is difficult if not impossible to obtain by other methods. The last parameter which Mills et al. [14] used was the square root of $k_{43}$. The square root was used to ensure positive values for $k_{42}$ and $k_{43}$. The reader should note that the parameter set chosen by Mills et al. [14] is only one of a large number of possible parameter sets.

Fig. 2 and table 2 show the expected systematic errors in the evaluation of the tetrameric Adair constants if a oxygen binding experiment were performed on hemoglobin $A_0$ at 1.0 and 3.0 mM heme, under specific solution conditions, and analyzed by the tetramer Adair formulation (eq. 12). It is expected that the free energy to bind the third oxygen will contain a systematic error of

0.40 kcal/mol at 1.0 mM, and 0.23 kcal/mol at 3.0 mM heme. This would lead to the conclusion that the triply liganded hemoglobin tetramer is present at much lower concentration than is actually the case.

Is the underestimation of the concentration of triply liganded species a general phenomenon which can be expected for all hemoglobin solutions and conditions, or is it specific to the particular distribution of equilibrium constants defined in the first column of table 1? The values of $\delta\Delta g_{43}$ and $\delta\Delta G_{43}$ for the Kansas data (fig. 3 and table 3) are in the other direction. This indicates that the triply liganded species for some distributions of Adair constants may be underestimated or overestimated for other such distributions.

Can an investigator use the concentration dependence of the median ligand concentration to predict what hemoglobin concentration is 'high' enough to allow the use of the tetramer Adair formulation? No! The median ligand concentration is proportional to the fourth root of the $K_{44}$. In table 3 the values of $\delta\Delta G_{44}$ for hemoglobin Kansas are $-0.48$ and $-0.29$ kcal/mol at 1.0 mM heme respectively. These correspond to changes in the median ligand concentration of 19 and 11%. This expected shift is clearly shown in the first figure of the paper by Atha et al. [16]. However, the values of $\delta\Delta G_{44}$ for hemoglobin $A_0$ in table 2 are $-0.06$ and $-0.03$ kcal/mol, respectively. This corresponds to a shift in the median ligand concentration of 2.5 and 1.3% for hemoglobin $A_0$ between 1.0 and 3.0 mM heme. Consequently, an overall measure of affinity, such as the median ligand concentration, cannot be used reliably as a method to predict the hemoglobin concentration that is high enough to allow the use of the tetramer Adair formulation.

Can an investigator use an increase in the variance of fit, $\sigma^2$, as a diagnostic tool to determine the hemoglobin concentration that is high enough to allow the use of the tetramer Adair formulation? No! A typical $\sigma^2$ for an actual experiment is on the order of $2 \times 10^{-5}$ in fractional saturation units and the values given in tables 2 and 3 are several orders of magnitude lower than what is experimentally observed. This indicates that the systematic errors which we predict will not cause an increase in the variance with actual experimental data. These systematic errors arise because the presence of dimers slightly alters the shape of the binding isotherm. When these data are analyzed by a tetramer Adair formulation, the slightly altered data can be fitted with excellent precision, but generate a systematic perturbation of the Adair constants.

How accurate do the Adair constants need to be? Or, are the induced systematic errors shown in tables 2 and 3 significant? The quaternary enhancement effect for hemoglobin $A_0$ is 0.81 kcal/mol [29]. Ackers and co-workers reported the value of the free energy to bind the second oxygen to a hemoglobin $A_0$ $\alpha\beta$ dimer as $-8.38$ kcal/mol [14], $-8.34$ kcal/mol [15] and $-8.35$ kcal/mol [17]. In a recent paper, Philo and Lary [8] have used a kinetic method to suggest that the value is $-8.66$ kcal/mol. Obviously, in these studies a difference of a few tenths of a kcal/mol must be considered as significant. If we refer to tables 2 and 3 it is clear that even at 3.0 mM heme the systematic errors induced by neglecting dimers can be a few tenths of a kcal/mol and must be considered as significant.

It is evident that significant systematic errors can occur if the dimeric species are neglected. It is particularly important to note that the induced systematic errors which are expected for hemoglobin $A_0$ are quite different from those for hemoglobin Kansas. This means that these results cannot be used to predict the results of neglecting dimeric species for other hemoglobins or simply changes in buffer conditions. Furthermore, overall measures of ligand affinity, such as the median ligand concentration, cannot be reliably used as indicators of when this problem will occur. Finally, the direction and magnitude of these systematic errors cannot be predicted a priori without having previously determined all of the equilibrium constants for the linkage scheme (fig. 1).

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