

## RESOLVABILITY OF ADAIR CONSTANTS FROM OXYGENATION CURVES MEASURED AT LOW HEMOGLOBIN CONCENTRATION \*

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Analyses of low concentration oxygenation curves for apparent Adair constants in which the effects of dimers is ignored have been explored using recently determined values of the overall energy coupling parameters. For high affinity systems and favorable energy distributions, it is found that the errors in estimated binding free energies may be less than one kcal provided the measurement errors are strictly random and of small magnitude. These errors are nevertheless quite substantial as compared with the differences between values for the successive binding steps.

### 1. Introduction

An extensive body of apparent Adair constants has been derived from oxygenation curves determined for human hemoglobin using the automatic oxygenation technique of Imai [1–5]. The method is most conveniently adapted for use with dilute hemoglobin solutions (e.g. 60  $\mu$ M heme) where appreciable dimers may be present. Since the dimeric species have higher oxygen binding affinity than the tetramers of interest it is important to evaluate the possible effects of dimeric species upon the shapes of measured curves and upon estimation of the Adair constants from experimental data. In order to do so it is necessary to know: (a) the free energies of dimer–tetramer association in both the unliganded and fully oxygenated states (their difference being the overall *free energy coupling*), (b) the partitioning of this coupling energy into parts associated with the successive binding steps, and (c) the free energy for binding four oxygens onto tetramers.

In a recent study [6] we explored by numerical analysis the resolvability of Adair constants by fitting low concentration simulated oxygenation curves to the tetramer Adair equation, when the simulations were based upon the best available estimate of the total free energy coupling, 8 kcal [7,8]. For a wide range of

energy distributions (i.e. different ways of partitioning the 8 kcal with respect to the binding steps) the estimation of Adair constants was found to be seriously perturbed by the presence of dimers. The degree of perturbation is highly dependent upon the dimer–tetramer association constant for fully oxygenated hemoglobin, and the overall oxygen affinity of tetramers (item (c) above). In the previous study most of the analyses pertained to hemoglobin systems of “medium affinity” (i.e.  $P_{50} \approx 15$  mmHg) and large oxygenation-linked subunit dissociation energy (8 kcal).

Recently we have developed a method for accurate determination of the total free energy coupling [9] and found it to be only 6.3 kcal in 0.1 M Tris HCl, 0.1 M NaCl, 1 mM Na<sub>2</sub>EDTA, pH 7.4, 21.5°C (a “high affinity” system with  $P_{50} \approx 5$  mm Hg for tetramers). Under these same conditions we have succeeded in determining the complete set of equilibrium constants for oxygen binding and subunit dissociation [10,11]. The free energies are listed in table 1, case I. Since the actual free energy coupling has been found to be substantially lower than the previous estimates [7,8], it seemed likely that a decreased error in estimation of Adair constants from oxygenation curves at low concentration would result. Consequently we have investigated the effects of varying the total free energy coupling upon these determinations. The results indicate that when the energy coupling is below 7 kcal the errors in estimation of apparent tetramer binding free energies

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Table 1  
Free energies used in generating simulated oxygenation curves <sup>a)</sup>

Case	Subunit association		O <sub>2</sub> binding to dimers		O <sub>2</sub> binding to tetramers			
	<sup>0</sup> ΔG <sub>2</sub>	<sup>4</sup> ΔG <sub>2</sub>	ΔG <sub>21</sub>	ΔG <sub>22</sub>	ΔG <sub>41</sub>	ΔG <sub>42</sub>	ΔG <sub>43</sub>	ΔG <sub>44</sub>
I	-14.38	-8.05	-8.78	-7.97	-6.26	-5.51	-7.56	-7.85
II	-16.05	-8.05	-9.20	-8.39	-6.02	-5.48	-7.46	-8.22
III	-15.05	-8.05	-8.94	-8.13	-6.16	-5.41	-7.62	-7.99
IV	-13.55	-8.05	-8.57	-7.76	-6.39	-5.62	-7.51	-7.66
V	-14.38	-8.05	-7.99	-7.18	-5.47	-4.16	-7.31	-7.05

<sup>a)</sup> Notation used has been defined previously [12]. <sup>0</sup>ΔG<sub>2</sub> and <sup>4</sup>ΔG<sub>2</sub> are dimer–tetramer association energies for unliganded and fully oxygenated hemoglobin, respectively, and are expressed in kcal/mole dimer. ΔG<sub>21</sub> and ΔG<sub>22</sub> are free energies for sequential oxygen binding steps to dimer. ΔG<sub>41</sub> pertain similarly to tetramer. The free energies correspond to equilibrium constants uncorrected for statistical factors.

δΔG<sub>4i</sub> from oxygenation data at 60 μM heme do not exceed 1 kcal provided the measurement errors are strictly random and of small magnitude. Previous conclusions regarding the devastating effects of the 8 kcal linkage are still valid, and are particularly applicable to low affinity systems.

## 2. Methods

Simulated oxygenation curves were generated on the basis of the generalized binding equation for the dimer–tetramer linkage system [12] and were analyzed in terms of the tetramer Adair equation. Numerical methods for simulation and analysis of oxygenation curves were the same as described previously [6]. Values of the free energies used are listed in table 1. Case I is the set of experimentally derived energies mentioned earlier [10,11]. Cases II–IV represent variations in the overall coupling energy while maintaining the same fractional partitioning of this energy with respect to successive binding steps, the same value of <sup>4</sup>ΔG<sub>2</sub>, and the same overall tetramer oxygen binding energy (−27.18 kcal/4 moles O<sub>2</sub>). Case V represents a lowered tetramer binding energy (−24 kcal). We have also explored the effect of decreasing <sup>4</sup>ΔG<sub>2</sub> so that substantially more dimeric species are created.

## 3. Results

Results are expressed in terms of free energies since these are generally the quantities of interest. From

values of the correct (i.e. originally assumed) Adair constants  $k_{4i}$  and the apparent constant  $k_{4i}^{app}$  derived from fitting to the tetramer Adair equation, the deviation δΔG<sub>4i</sub> is calculated as  $RT \ln(k_{4i}/k_{4i}^{app})$ .

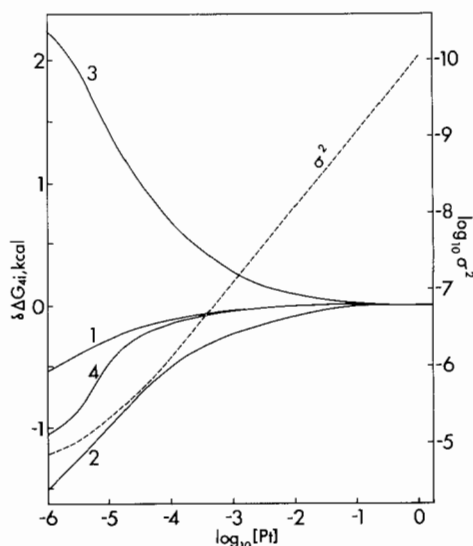


Fig. 1. Effect of hemoglobin concentration on resolvability of the successive binding energies for case I (table 1) where the overall free energy coupling is 6.34 kcal. Oxygenation curves were simulated without errors according to the generalized isotherm which takes into account the dissociation of tetramers into dimers. Resulting error-free curves were then analyzed according to the tetramer Adair equation for apparent Adair constants  $K_{4i}^{app}$ . The deviation in free energy from correct values (listed in table 1) plotted above are calculated as  $\Delta G_{4i} = RT \ln(k_{4i}/k_{4i}^{app})$  where  $k_{4i}$  are the true values.

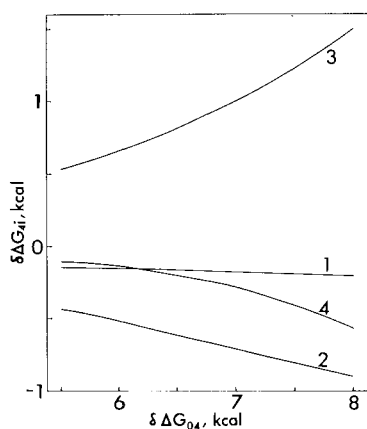


Fig. 2. Effect of overall energy coupling upon resolvability of the successive binding energies at a hemoglobin concentration of  $60 \mu\text{M}$  heme. The free energy coupling  $\delta\Delta G_{04}$  represents the difference in subunit dissociation free energy between unliganded and fully liganded species. Values of all energies used are listed in table 1 (cases I–V).

### 3.1. Effect of total hemoglobin concentration ( $P_T$ )

The effect of varying total hemoglobin concentration upon resolvability of the Adair constants is shown in fig. 1 for the system we have experimentally solved (case I, table 1). It can be seen that the errors in binding energy do not exceed 0.5 kcal for concentrations above approximately  $10^{-4}$  M heme and that  $k_{41}$  and  $k_{44}$  are generally resolved much better than  $k_{42}$ ,  $k_{43}$ . For concentrations of  $60 \mu\text{M}$  the error on  $k_{43}$  is seen to be approximately 1 kcal and to exceed 2 kcal at  $1 \mu\text{M}$  heme. The variance of the fit shown in fig. 1 is a measure of the systematic errors induced by neglecting dimeric species.

### 3.2. Effect of overall free energy coupling

The effect of variations in total coupling energy  $\delta\Delta G_{04}$  is shown in fig. 2 for a concentration of  $60 \mu\text{M}$  heme. As the coupling energy increases from 5.5 kcal to 8 kcal the maximum error in estimated energies is seen to increase from 0.5 kcal to 1.5 kcal for this case.

### 3.3. Effect of decreasing affinity

When the affinity of tetramers is decreased to  $-24$  kcal/4 oxygens, while using the same  $\delta\Delta G_{04}$  of

6.34 kcal (case V, table 1) the resolvability of Adair constants decreases dramatically. The values of  $\delta\Delta G_{4i}$  at  $60 \mu\text{M}$  heme are:  $\delta\Delta G_{41} = -0.1$  kcal,  $\delta\Delta G_{42} = -1.5$  kcal,  $\delta\Delta G_{43} = 0.5$  kcal,  $\delta\Delta G_{44} = 0.2$  kcal. Previous calculations have shown the much greater errors for such low affinity systems based on 8 kcal overall energy coupling [6].

### 3.4. Variation of ${}^4\Delta G_2$

Similarly when the dimer–tetramer association energy is decreased so that more dimers are produced while maintaining the same energy coupling (i.e.  ${}^0\Delta G_2 = -13.98$  kcal,  ${}^4\Delta G_2 = -7.64$  kcal) the value of  $\delta\Delta G_{42}$  becomes  $-0.94$  kcal (as compared with  $-0.74$  for case I, table 1).

### 3.5. Effects of random and systematic errors

The calculations presented here are based upon simulated oxygenation curves which are error-free (i.e. the variance is  $<10^{-20}$ ). Consequently the increasing variance of fits shown in fig. 1 represent systematic errors induced by fitting to a model which does not consider dimeric species. It should be noted that in the neighborhood of  $60 \mu\text{M}$  heme these induced systematic errors became quite comparable in magnitude with the random measurement errors corresponding to the highest precision measurements [1–5]. When measurement errors are then added to the simulated curves the confidence intervals for the fits become substantially wider. Any additional systematic errors (e.g. instrumental artifacts) may have the effect of either augmenting or smoothing out the small shape perturbations arising from the presence of dimeric species.

## 4. Conclusions

Results of this study show that for a hemoglobin system undergoing an oxygenation-linked dimer–tetramer equilibrium the dimer-induced errors to be expected in resolving apparent tetramer binding energies may be substantially lower than indicated by previous calculations. This is a combined consequence of (a) a smaller free energy coupling, (b) a higher oxygen affinity and (c) a larger free energy for liganded subunit association. The free energy errors are nevertheless quite

substantial as compared with the differences between values for the successive binding steps. For the experimental system represented by case I, for example, the Wyman interaction energy  $\Delta G_I (= \Delta G_{44} - \Delta G_{41})$  is only 2.8 kcal. The binding energies  $\Delta G_{44}$  and  $\Delta G_{41}$  are corrected for statistical factors in this calculation, maximizing their difference [11].

The analyses presented here for "high affinity" hemoglobin systems, taken together with the previous analysis of "medium affinity" cases [6] provide a comprehensive picture of results to be expected when the dissociated dimers are noncooperative. This condition has been found to be fulfilled by the hemoglobin systems analyzed to date [10,11].

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