Resolvability of free energy changes for oxygen binding and subunit association by human hemoglobin

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ABSTRACT Probability distributions of the free energy changes for oxygen binding, subunit association, and quaternary enhancement by human hemoglobin were obtained from Monte Carlo simulations performed on two independent sets of variable protein concentration equilibrium oxygen-binding data. Uncertainties in unliganded and fully liganded dimer to tetramer association free energy changes ($^0\Delta G_2'$ and $^4\Delta G_2'$) were accounted for in the simulations. Distributions of the dimer to tetramer association free energy changes for forming singly and triply liganded tetramers ($^1\Delta G_2'$ and $^3\Delta G_2'$) are well defined and quite symmetric, whereas that for forming doubly liganded tetramers ($^2\Delta G_2'$) is poorly defined and highly asymmetric. The distribution of the dimer stepwise oxygen-binding free-energy change ($\Delta g_{2i}'$) is well defined and quite symmetric as are those of the tetramer stepwise oxygen-binding free-energy changes for binding the first and last oxygens to tetramers ($\Delta g_{41}'$ and $\Delta g_{44}'$). Distributions of the intermediate tetramer stepwise oxygen-binding free-energy changes ($\Delta g_{42}'$ and $\Delta g_{43}'$) are poorly defined and highly asymmetric, but are compensatory in that their sum ($\Delta g_{4[2+3]}'$) is again well defined and nearly symmetric. Distributions of the free energy changes corresponding to the tetramer product Adair oxygen binding constants ($\Delta G_{4i}'$) are well defined and quite symmetric for $i = 1, 3, 4$ but not for $i = 2$. The distribution of $\Delta g_{44}' - \Delta g_{2i}'$ (the quaternary enhancement free energy change) is relatively narrow, nearly symmetric, and confined to the negative free-energy domain. This suggests that the quaternary enhancement free energy change (*a*) may be resolved with good confidence from this data and (*b*) is finite and negative under the conditions of these experiments. Our results also suggest two different four-state combinatorial switch models that provide accurate characterization of hemoglobin's functional behavior.

INTRODUCTION

Recently, there has been some controversy as to the magnitude of the quaternary enhancement free energy change exhibited by human hemoglobin. Specifically, quaternary enhancement refers to enhanced affinity of triply-liganded hemoglobin tetramers for binding oxygen to their last available site relative to the affinity of singly-liganded hemoglobin dimers for binding oxygen to their last available site. The quaternary structural interactions arising from association of a singly-liganded dimer and a doubly-liganded dimer to form a triply-liganded tetramer thus produce enhanced affinity in the tetramer relative to the dissociated dimers for binding the fourth oxygen to a system defined in this way. Chu et al. (1984) and Mills et al. (1976) have determined from analysis of equilibrium oxygen binding data obtained over a range of hemoglobin concentrations that ~ -0.5 kcal/mol of a quaternary enhancement free-energy change is displayed by human hemoglobin A_0. Gibson and Edelstein (1987) have argued that, within experimental error, there is no evidence for quaternary enhancement. On the other hand, the results of Gill et al. (1987) imply an infinitely negative quaternary enhancement free energy change as a consequence of their claim of effectively no triply-liganded tetramers over the course of oxygenation of human hemoglobin. The controversy spawned by these reports arises primarily as a result of the inherent difficulty encountered when estimating confidence limits of fitting parameters characteristic of nonlinear models. The process of confidence interval estimation, no matter how sophisticated, requires implementation of at least some approximations (Johnson and Frasier, 1985; Straume and Johnson, 1988; Straume et al., 1989). To address this controversy regarding the quaternary enhancement free energy change, we have characterized the resolvability of various free energy change parameters of human hemoglobin.

In order to test the resolvability of the parameters characteristic of oxygen binding and subunit association by human hemoglobin, we have employed a Monte Carlo approach (Bard, 1974; Press et al., 1986) in which we have accounted for experimental uncertainties encoun-

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0006-3495/89/07/15/11 $2.00

tered in two independent sets of equilibrium oxygen binding data obtained over a range of protein concentrations. This procedure permits explicit derivation of any desired parameter's probability distribution function thus obviating the need to invoke most of the approximations needed for determining parameter confidence intervals by the classical methods. Knowledge of the full form of any parameter's probability distribution function, indicative of the resolvability of this parameter's value, thus contributes to the confidence one may have in a model-dependent interpretation that relies on that particular value of an oxygen-binding or subunit-association free-energy change. Because of the abundance of models for hemoglobin action that have been proposed over the years, reliance upon a single derived value for a particular parameter sometimes can be a dangerous practice if the uncertainty in that parameter value is great. In other words, multiple replicate experiments may yield widely variable values for some of the parameters characteristic of hemoglobin function thus making accurate interpretation difficult or invalid without knowledge of the full probability distribution functions. The probability distributions obtained by the present study contribute information useful for interpretation regarding hemoglobin's cooperative interactions by explicitly addressing the resolvability of free energy change parameters characteristic of oxygen binding and subunit association by human hemoglobin.

MATHEMATICAL PROCEDURES

The thermodynamic linkage scheme characteristic of hemoglobin oxygen binding and subunit association (Ackers and Halvorson, 1974; Johnson et al., 1976; Straume and Johnson, 1988) involves free energy changes descriptive of (*a*) the two oxygenation steps of hemoglobin dimers ($\Delta g'_{2i}$) representing the intrinsic oxygen-binding free-energy changes for forming *i*-liganded from $(i-1)$-liganded dimers ($\Delta g'_{21} = \Delta g'_{22}$, assuming no cooperativity in hemoglobin dimers), (*b*) the four oxygenation steps of hemoglobin tetramers ($\Delta g'_{4i}$) representing the intrinsic oxygen-binding free-energy changes for forming *i*-liganded from $(i-1)$-liganded tetramers, and (*c*) the five subunit association processes (${}^{i}\Delta G'_2$) representing the intrinsic dimer to tetramer association free energy changes for forming *i*-liganded tetramers from appropriately liganded dimers. However, it should be noted that only seven of these free energy changes are independent because the linkage scheme includes several closed circular pathways (Ackers and Halvorson, 1974). The parameters described here are the intrinsic oxygen binding and subunit association free energy changes (denoted with the prime (') notation) and thus do not include contributions from statistical degeneracies that arise as a result of multiple possible distributions of oxygen among the subunits of dimers or tetramers.

A minimum of six mathematically independent parameters from among the eleven above mentioned parameters is required to fully characterize the oxygen binding and subunit association linkage scheme. Seven would be required if hemoglobin dimers were assumed to exhibit some form of cooperativity, i.e., if $\Delta g'_{21} = \Delta g'_{22}$ did not hold. The six mathematically independent parameters actually employed in nonlinear least squares estimation were those reported by Johnson et al. (1976): ${}^{0}\Delta G'_2$, ${}^{4}\Delta G'_2$, $\Delta G'_{44}$, $(k_{43})^{1/2}$, ${}^{0}K_2/{}^{1}K_2$, and ${}^{3}K_2/{}^{4}K_2$. ${}^{0}\Delta G'_2$ and ${}^{4}\Delta G'_2$ were determined independently for each of the data sets employed in these studies and we included standard deviations of 0.1 kcal/mol for each of these parameters (as suggested from the original data). The remaining four parameters were those varied by the modified Gauss-Newton nonlinear least squares estimation algorithm employed here (Johnson and Frasier, 1985). The free energy change for forming fully liganded tetramers from unliganded tetramers is represented by $\Delta G'_{44}$. The free energy change for forming triply-liganded tetramers from doubly-liganded tetramers is contained in $(k_{43})^{1/2} = [\exp(-\Delta g_{43}/RT)]^{1/2}$. The parameters ${}^{0}K_2/{}^{1}K_2 = \exp[-(\Delta g_{21} - \Delta g_{41})/RT]$ and ${}^{3}K_2/{}^{4}K_2 = \exp[-(\Delta g_{22} - \Delta g_{44})/RT]$ represent two remaining mathematically independent parameters that permit unique characterization of the full oxygen binding and subunit association linkage scheme of human hemoglobin.

The original variable protein concentration equilibrium oxygen binding data of Chu et al. (1984) and Mills et al. (1976) were supplied by Dr. Gary K. Ackers. The experimental conditions for these studies were 0.1 M Tris, 0.1 M NaCl, 1.0 mM Na_2EDTA, pH 7.4 at 21.5°C. The buffer conditions employed in these experiments were chosen to approximately reproduce the native physiological environment of human hemoglobin. The data of Chu et al. (1984) consisted of four individual oxygen binding experiments at hemoglobin concentrations of 5.15, 10.0, 14.12, and 27.1 μM for a total of 283 data points. The data of Mills et al. (1976) included 236 total data points and were obtained at hemoglobin concentrations of 5.355, 38.25, 76.5, and 382.5 μM (two experiments were conducted at 76.5 μM). The range of fractional oxygen saturation values encountered in these data sets ranged from <0.005 to >0.995. The range of oxygen concentrations considered in these experiments spanned from 9.3×10^{-8} to 2.9×10^{-4} M for the data of Chu et al. (1984) and from 2.7×10^{-7} to 2.7×10^{-4} M for the data of Mills et al. (1976). In addition to the oxygen binding data each of these data sets includes an independent experimental evaluation of the unliganded (${}^{0}\Delta G'_2$) and fully liganded (${}^{4}\Delta G'_2$) dimer to tetramer association free energy changes that were simultaneously evaluated on the same hemoglobin preparation and under the same experimental conditions.

The data sets of Chu et al. (1984) and Mills et al. (1976) were specifically chosen for this study because they include a variation in both oxygen concentration and hemoglobin concentration. The primary objective here is to investigate the uncertainties in experimentally observed parameters, such as the quaternary enhancement effect, which reflect differences in the oxygen binding properties of the dimeric and tetrameric oligomers of hemoglobin A_0. In order to investigate such parameters it is a requirement that the data sets contain information on (*a*) the oxygen binding properties of hemoglobin tetramers, (*b*) the oxygen binding properties of hemoglobin dimers, and (*c*) the dimer to tetramer subunit assembly process. In effect what we (Mills et al., 1976) did was to collect data in two dimensions, i.e., oxygen and hemoglobin concentrations, instead of only a single dimension, i.e., oxygen concentration. The process, and the increase in resolvability, is analogous to using two dimensional Nuclear Magnetic Resonance or electrophoresis instead of their one dimensional counterparts. The data sets of Chu et al. (1984) and Mills et al. (1976) are the only two dimensional oxygen binding and subunit assembly data sets that are available for hemoglobin A_0.

Each of the fractional oxygen saturation values was assigned an equal weighting factor during analysis. The actual uncertainty associated with the values will increase slightly as the fractional saturation approaches zero or one, however, the actual variation in these weighting factors was calculated to be <5% for the data considered in this work. A recent report indicating that the extinction coefficients of the variously ligated forms of hemoglobin tetramers vary, particularly the intermediately ligated forms (Doyle et al., 1988), suggests that the values of Adair constants derived from these equilibrium oxygen binding data will be in

error by a few precent. However, a few percent shift in the values of the derived Adair constants will not alter the general conclusions reported here. A 3% error in the derived Adair constants will produce a shift of 0.017 kcal/mol in the corresponding free energy change. Errors of this magnitude in the values for the free energy change parameters considered here will not have a significant effect on any of the conclusions drawn from this work.

Nonlinear least squares estimation of the above mentioned parameters was performed to independently determine the best fit set of parameter values characteristic of each of these data sets. The values derived in this way were used to synthesize noise-free fractional oxygen saturation data at the same oxygen and protein concentrations encountered in the original experiments. The square root of the variance of fit obtained from this initial parameter estimation of the original data was used as an estimate of the uncertainty (standard deviation) present in the fractional oxygen saturation data points of each respective data set. Clearly, by employing such an approach, we are assuming that no systematic behavior is present in the original data that is not fully and accurately accounted for by the model used in analysis. For the purposes of this study, i.e., to test the resolvability of the various free energy changes characteristic of human hemoglobin oxygen binding and subunit association for the buffer conditions mentioned above, we generated 500 different sets of Gaussian distributed noise (the width of which was defined by the estimated standard deviation) to superimpose on the calculated noise-free data. Gaussian distributed uncertainties in ${}^0\Delta G_2'$ and ${}^4\Delta G_2'$ (standard deviation of 0.1 kcal/mol) were also included in the Monte Carlo simulations based on estimates from the original experiments. Uncertainties in these parameters were not explicitly considered in the original reports. Nonlinear least squares parameter estimations were then performed on each of the five hundred sets of data in which ${}^0\Delta G_2'$ and ${}^4\Delta G_2'$ were rigidly constrained to their input values and $\Delta G_{44}'$, $(k_{43})^{1/2}$, ${}^0K_2/{}^1K_2$, and ${}^3K_2/{}^4K_2$ were employed as fitting parameters. The results of each parameter estimation were recorded independently. After completing the 500 estimations for each data set, 51-interval histograms were generated representing the probability distributions of the various free energy changes of interest. The resultant histograms were then used to estimate the most probable derived parameter values and lower and upper 67% confidence intervals for each free energy change parameter considered. Most probable values were estimated by the following equation:

$$\text{value (mp)} = \frac{\sum_{i=1}^{51} (\text{rel prob})_i^{11.358}\ \text{value}_i}{\sum_{i=1}^{51} (\text{rel prob})_i^{11.358}}.$$

The power of 11.358 was selected because relative probabilities of 0.67 or less will contribute 0.01 or less to this weighted average (i.e., $[\log(1/100)/\log(2/3)] = 11.358$ such that $(2/3)^{11.358} = (1/100)$). Only those values with associated relative probabilities of 0.67 or more will contribute more than 1% to the weight average and thus a reasonable estimate for the most probable parameter value is produced.

The procedure outlined in the preceding paragraph is a minor modification of a standard Monte Carlo procedure (Press et al., 1986; Bard et al., 1974) for the evaluation of uncertainty distributions of estimated parameters. The modification was to include a distribution of uncertainties in the values of ${}^0\Delta G_2'$ and ${}^4\Delta G_2'$, thus precluding the requirement that these parameters be assigned fixed values when they are only known to limited precision. By including this modification we considered the simultaneous variation of six of the required seven independent linkage parameters in our evaluation of the distributions of uncertainties. To obtain the seventh independent linkage parameter we assumed that dimeric hemoglobin binds oxygen without cooperativity, as has been experimentally observed (Mills et al., 1976; Chu et al., 1984).

RESULTS AND DISCUSSION

The recent controversy regarding the magnitude of the quaternary enhancement free energy exhibited by human hemoglobin (Gibson and Edelstein, 1987; Gill et al., 1987) has prompted us to consider the resolvability of the free energy changes characteristic of oxygen binding and subunit association by this cooperative macromolecular protein assembly. Model-dependent interpretation of hemoglobin's functional behavior relies upon knowledge of the free energy changes associated with the various steps of the thermodynamic linkage scheme (see Mathematical Procedures). Uncertainties in the values of some of these parameters can have critical implications regarding the validity of particular mechanistic models of hemoglobin action. It is often difficult, however, to accurately estimate the confidence intervals of fitting parameters descriptive of nonlinear mathematical models. Approximations are implicit in even the most sophisticated of methods (Johnson and Frasier, 1985; Straume and Johnson, 1988; Straume et al., 1989). Particularly challenging is the extrapolation of reliable estimates of confidence intervals associated with composite free energy changes that are calculated from confidence intervals estimated for constituent parameters. For example, the quaternary enhancement free energy change exhibited by human hemoglobin is defined as the difference between the intrinsic tetramer stepwise oxygen binding free energy change for binding the last oxygen to hemoglobin tetramers ($\Delta g_{44}'$) and the intrinsic stepwise oxygen binding free energy change for binding the last oxygen to hemoglobin dimers ($\Delta g_{22}'$), i.e., $\Delta g_{44}' - \Delta g_{22}'$. In order to reliably estimate the confidence intervals associated with the quaternary enhancement free energy change, it would be necessary to estimate this parameter directly in the analysis process. Then confidence intervals could be determined on this particular parameter by the estimation algorithm, thus directly and appropriately accounting for correlations that may influence the derived confidence intervals (Johnson and Frasier, 1985; Straume and Johnson, 1988; Straume et al., 1989). If the uncertainty in the quaternary enhancement free energy change is estimated from the uncertainties associated with the two free energy changes from which it is derived (i.e., $\Delta g_{44}'$ and $\Delta g_{22}'$), information regarding cross correlation among parameters is lost unless painstaking attempts are made to accurately map the variance spaces of the respective constituent parameters ($\Delta g_{44}'$ and $\Delta g_{22}'$) onto the space associated with the parameter of interest ($\Delta g_{44}' - \Delta g_{22}'$). The Monte Carlo approach that we have employed in the studies reported here implicitly retains all information concerning correlations among independent free energy change parameters.

TABLE I Results of Monte Carlo simulations performed on the equilibrium oxygen binding experiments of Chu et al. (1984) and Mills et al. (1976)*

Data	Initial	$P(<In)$	$P(>In)$	MP	−(67%)	+(67%)	$P(<0)$	$P(>0)$
Variance × 10^6								
Chu	11.948	0.304	0.696	12.109	0.886	2.090	—	—
Mills	25.661	0.290	0.710	26.264	2.262	4.396	—	—
$^0\Delta G'_2$								
Chu	−14.421	0.481	0.519	−14.410	0.094	0.091	—	—
Mills	−14.437	0.538	0.462	−14.469	0.079	0.111	—	—
$^4\Delta G'_2$								
Chu	−8.091	0.473	0.527	−8.092	0.083	0.092	—	—
Mills	−8.078	0.516	0.484	−8.140	0.067	0.132	—	—
$^1\Delta G'_2$								
Chu	−11.675	0.421	0.579	−11.673	0.079	0.101	—	—
Mills	−11.521	0.357	0.643	−11.498	0.082	0.091	—	—
$^2\Delta G'_2$								
Chu	−6.969	0.373	0.627	−8.586	0.186	6.426	0.802	0.198
Mills	−8.457	0.486	0.514	−9.026	0.205	1.984	0.922	0.078
$^3\Delta G'_2$								
Chu	−7.489	0.428	0.572	−7.499	0.079	0.125	—	—
Mills	−7.731	0.622	0.378	−7.839	0.050	0.183	—	—
$\Delta g'_{2l}$								
Chu	−8.324	0.489	0.555	−8.326	0.027	0.031	—	—
Mills	−8.411	0.514	0.486	−8.411	0.029	0.028	—	—
$\Delta g'_{41}$								
Chu	−5.578	0.262	0.738	−5.566	0.029	0.033	—	—
Mills	−5.495	0.234	0.766	−5.474	0.035	0.058	—	—
$\Delta g'_{42}$								
Chu	−3.618	0.373	0.627	−5.228	0.204	6.384	0.644	0.356
Mills	−5.347	0.506	0.494	−5.744	0.375	2.236	0.875	0.125
$\Delta g'_{43}$								
Chu	−8.844	0.624	0.376	−7.301	6.695	0.461	—	—
Mills	−7.685	0.527	0.473	−7.281	2.461	0.500	—	—
$\Delta g'_{44}$								
Chu	−8.925	0.450	0.550	−8.882	0.136	0.056	—	—
Mills	−8.760	0.380	0.620	−8.594	0.271	0.054	—	—
$\Delta g'_{4(2+3)}$								
Chu	−12.462	0.555	0.445	−12.500	0.064	0.129	—	—
Mills	−13.032	0.661	0.339	−13.129	0.090	0.196	—	—
$\Delta G'_{42}$								
Chu	−9.196	0.371	0.629	−10.816	0.160	6.429	0.896	0.104
Mills	−10.842	0.473	0.527	−11.329	0.191	1.920	0.939	0.061
$\Delta G'_{43}$								
Chu	−18.040	0.533	0.467	−18.078	0.039	0.118	—	—
Mills	−18.527	0.608	0.392	−18.687	0.029	0.242	—	—
$\Delta G'_{44}$								
Chu	−26.965	0.445	0.555	−26.959	0.027	0.027	—	—
Mills	−27.286	0.353	0.647	−27.281	0.016	0.020	—	—
$\Delta g'_{41} - \Delta g'_{2l}$								
Chu	2.746	0.363	0.637	2.768	0.052	0.044	—	—
Mills	2.916	0.245	0.755	2.943	0.044	0.067	—	—
$\Delta g'_{42} - \Delta g'_{2l}$								
Chu	4.706	0.374	0.626	3.087	0.218	6.391	—	—
Mills	3.064	0.505	0.495	2.557	0.300	2.125	—	—

TABLE I (Continued)

Data	Initial	$P(<In)$	$P(>In)$	MP	−(67%)	+(67%)	$P(<0)$	$P(>0)$
$\Delta g'_{43} - \Delta g'_{2i}$								
Chu	−0.520	0.624	0.376	0.998	6.681	0.467	0.674	0.326
Mills	0.726	0.528	0.472	1.101	2.467	0.514	0.351	0.649
$\Delta g'_{44} - \Delta g'_{2i}$								
Chu	−0.601	0.480	0.520	−0.557	0.169	0.077	—	—
Mills	−0.349	0.396	0.604	−0.196	0.288	0.086	0.996	0.004
$^1\Delta G'_2 - {}^0\Delta G'_2$								
Chu	2.746	0.363	0.637	2.768	0.052	0.044	—	—
Mills	2.916	0.245	0.755	2.943	0.044	0.067	—	—
$^2\Delta G'_2 - {}^0\Delta G'_2$								
Chu	7.452	0.372	0.628	5.808	0.182	6.336	—	—
Mills	5.980	0.489	0.511	5.456	0.261	2.061	—	—
$^3\Delta G'_2 - {}^0\Delta G'_2$								
Chu	6.932	0.431	0.569	6.959	0.110	0.109	—	—
Mills	6.706	0.580	0.420	6.616	0.074	0.195	—	—
$^4\Delta G'_2 - {}^0\Delta G'_2$								
Chu	6.331	0.497	0.503	6.284	0.121	0.165	—	—
Mills	6.357	0.468	0.532	6.379	0.130	0.124	—	—

*Initial = parameter value obtained from nonlinear least squares analysis of the original data; $P(<In)$, $P(>In)$ = probability that the distributions resulting from the Monte Carlo simulations produced a parameter value less than or greater than the initial value obtained from the analysis of the original data; MP = the estimated most probable parameter value produced by the Monte Carlo simulations; −(67%), +(67%) = the lower or upper 67% confidence intervals relative to the most probable value (MP) obtained from the Monte Carlo simulations; $P(<0)$, $P(>0)$ = the probability that the Monte Carlo simulations produced a parameter value less than or greater than zero; all free energy changes are in kcal/mol; the original equilibrium oxygen binding experiments considered here were conducted in 0.1 M Tris, 0.1 M NaCl, 1.0 mM Na_2EDTA, pH 7.4 at 21.5°C.

A complete summary of our results is presented in Table 1. We report there the results of parameter estimations performed on the original data (Initial), probabilities that the Monte Carlo simulations produced parameter values less than or greater than the initial best fit values to the original data ($P[<In]$ and $P[>In]$), the most probable values (MP) obtained from the simulations with their associated lower and upper 67% confidence intervals (−[67%] and +[67%]), and probabilities that the Monte Carlo simulations produced parameter values less than or greater than zero ($P[<0]$ and $P[>0]$).

In Fig. 1 are displayed histograms of the probability distributions for the variances of fit obtained from the simulations for both the data of Chu et al. (1984) (*solid line*) and Mills et al. (1976) (*dotted line*). The estimated most probable variances are somewhat greater than those obtained from analyses of the original data but the distributions are not very broad and only marginal asymmetry toward the high side is apparent. It was common for the most probable parameter values derived from the simulations to vary slightly from the values obtained from analysis of the original data. Agreement was generally within a few hundredths of a kcal/mol except in those cases where the probability distributions were very broad and asymmetric (i.e., those cases where the derived distributions spanned many kcal/mol).

Simulated probability distributions for the parameters $^0\Delta G'_2$ and $^4\Delta G'_2$, the dimer to tetramer subunit association free energy changes for forming unliganded and fully liganded tetramers, respectively, are presented in Fig. 2. These distributions were those generated by the simulation process and are approximately Gaussian distributed with standard deviations of 0.1 kcal/mol (as estimated from the original experiments). Both of these parameters were rigidly constrained at their simulated values during the parameter estimations performed here.

The derived probability distributions of the remaining three intrinsic dimer to tetramer subunit association free energy changes, $^1\Delta G'_2$, $^2\Delta G'_2$, and $^3\Delta G'_2$, are presented in Fig. 3. $^1\Delta G'_2$ and $^3\Delta G'_2$ are represented by relatively narrow probability distributions (ranging over <1 kcal/mol) that show only marginal asymmetry toward more positive free energy values. The probability distributions of $^2\Delta G'_2$, however, exhibit considerable asymmetry toward more positive free energy values and are quite poorly determined (the values range over as much as 20 kcal/mol). The difficulty in determining the magnitude of this parameter with confidence has been pointed out previously (Mills et al., 1976; Johnson et al., 1976).

The probability distributions of the intrinsic dimer stepwise oxygen-binding free-energy change, $\Delta g'_{2i}$, are presented in Fig. 4. We have assumed that hemoglobin

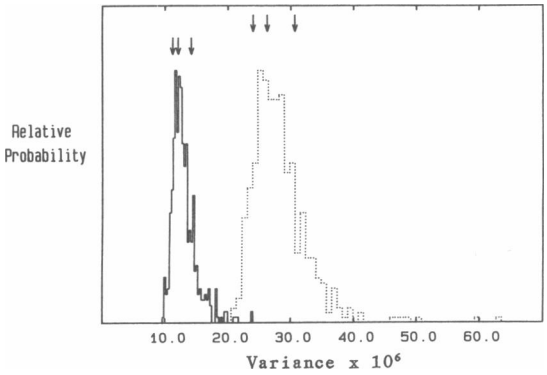

FIGURE 1 Derived variances of fit from simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). The values of the variance of fit presented here arise from data sets in which the uncertainty associated with each of the fractional oxygenation data points is equal and has a value of 1.0 (i.e., the reported values correspond to an equally weighted, unnormalized variance of fit). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

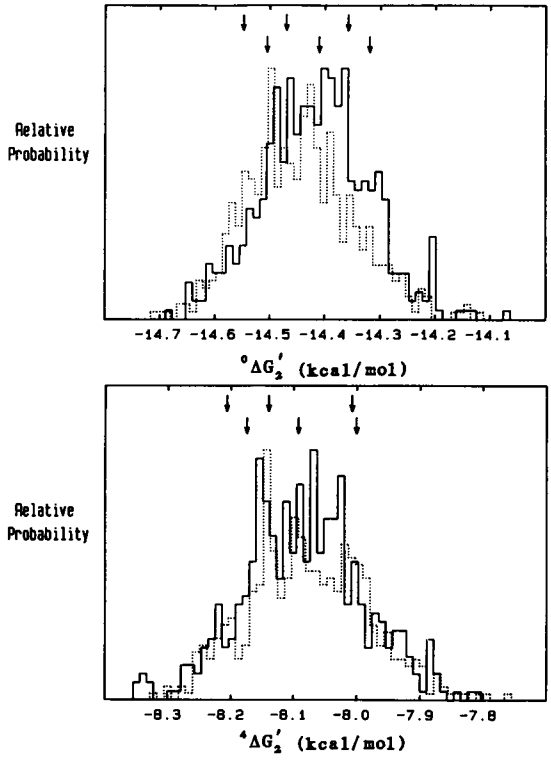

FIGURE 2 Simulated dimer to tetramer association free energies for forming unliganded ($^{0}\Delta G_2'$) and fully liganded ($^{4}\Delta G_2'$) tetramers for the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

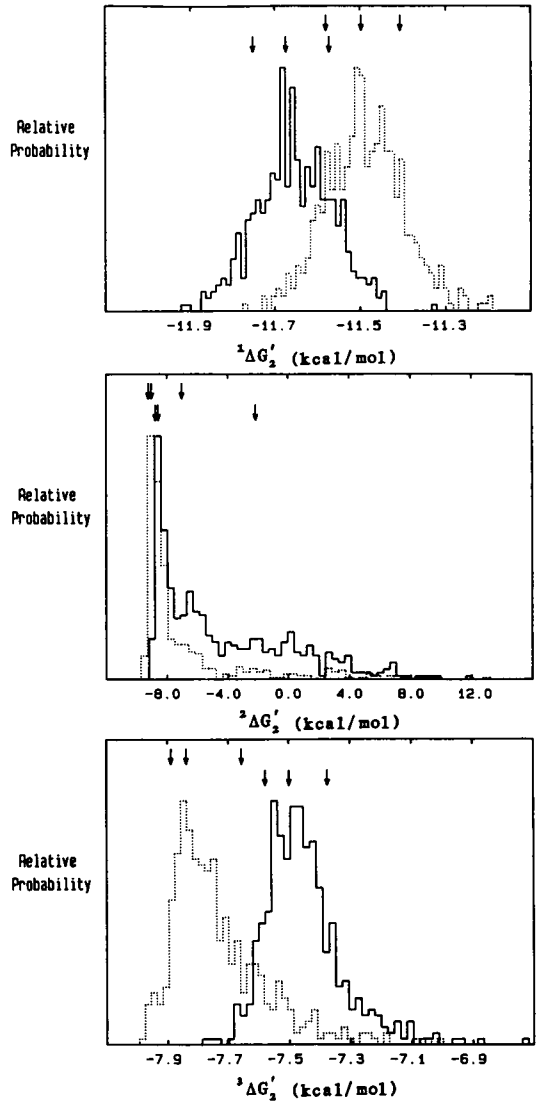

FIGURE 3 Derived intrinsic dimer to tetramer association free energy changes for forming singly ($^{1}\Delta G_2'$), doubly ($^{2}\Delta G_2'$), and triply ($^{3}\Delta G_2'$) liganded tetramers from appropriately liganded dimers for simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

dimers exhibit no cooperativity, therefore $\Delta g_{21}' = \Delta g_{22}'$ and both the first and second intrinsic dimer stepwise oxygen-binding free-energy changes are characterized by the same probability distribution. Both the Chu et al. (1984) data and the Mills et al. (1976) data produce nearly symmetric distributions which range in value over ~0.15 kcal/mol.

The probability distributions of the four intrinsic tetramer stepwise oxygen-binding free-energy changes, $\Delta g_{4i}'$, are displayed in Fig. 5. The free energy changes

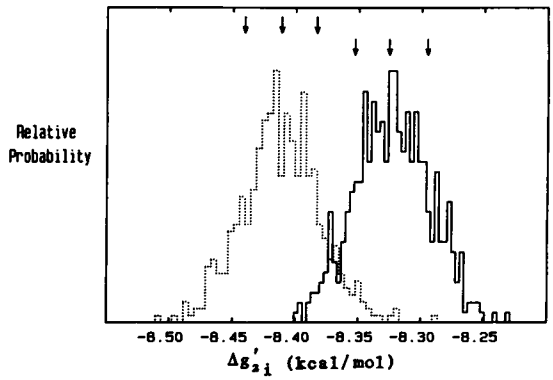

FIGURE 4 Derived instrinsic dimer stepwise oxygen-binding free-energy change ($\Delta g'_{2i}$, dimers assumed noncooperative) from simulations of the data of Chu et al. (1984), (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

associated with binding the first and last oxygens to hemoglobin tetramers, $\Delta g'_{41}$ and $\Delta g'_{44}$, respectively, are characterized by relatively narrow probability distributions that range over a few tenths of a kcal/mol in the case of $\Delta g'_{41}$ to ~1 kcal/mol for $\Delta g'_{44}$. The intermediate intrinsic tetramer stepwise oxygen-binding free-energy changes, $\Delta g'_{42}$ and $\Delta g'_{43}$, for binding the second and third oxygens to hemoglobin tetramers, respectively, are very poorly determined parameters as the probability distributions range over ~20 kcal/mol and are highly asymmetric. The distributions for $\Delta g'_{42}$ are seen to span well into the positive free energy domain suggesting that doubly liganded tetramers account for a vanishingly small fractional population of species occurring over the range of hemoglobin's oxygen saturation cycle. The distributions derived for $\Delta g'_{43}$ are also highly asymmetric but exhibit gradually decaying probability in the direction of more negative free energies. The asymmetries displayed by the probability distributions of these two intermediate tetramer oxygen-binding free-energy changes are therefore compensatory and, as can be seen by the distributions of the sums of these two intermediate parameters ($\Delta g'_{4(2+3)}$, see Fig. 6), the intrinsic free energy change associated with forming triply-liganded tetramers from singly-liganded tetramers is a well defined quantity (spanning only ~1 kcal/mol). This result indicates that it is not difficult to define the free energy change associated with adding the second and third oxygens to hemoglobin tetramers but it is difficult to determine how this free energy is distributed among these two consecutive steps.

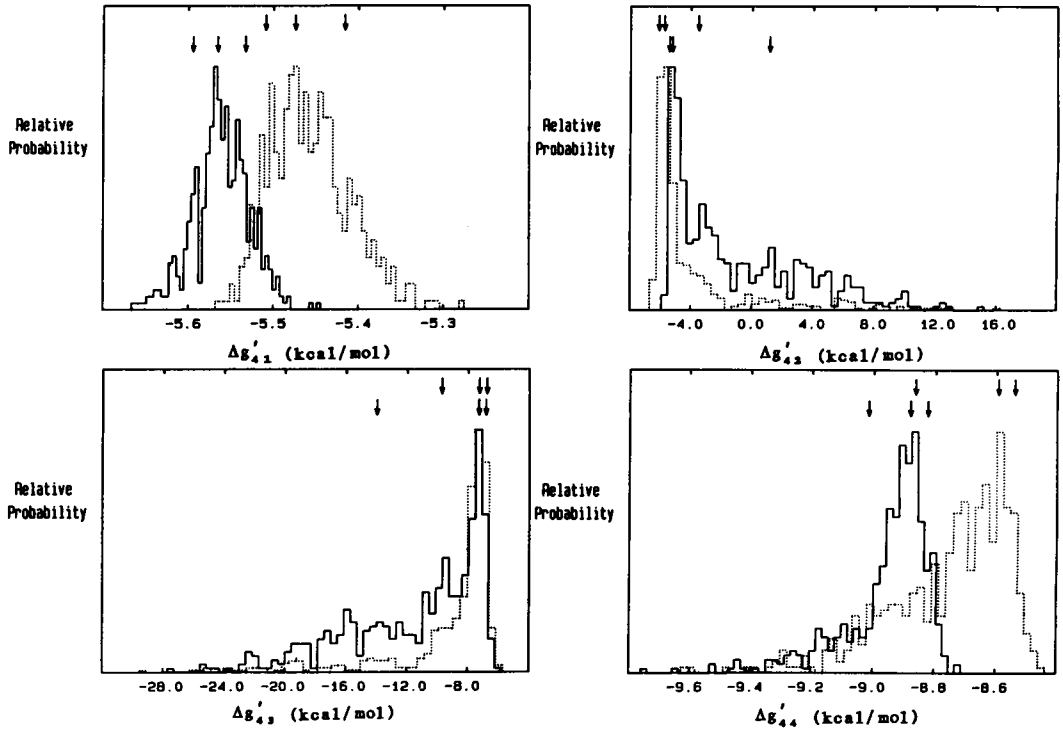

FIGURE 5 Derived intrinsic tetramer stepwise oxygen-binding free-energy changes ($\Delta g'_{4i}$, $i = 1, 2, 3, 4$) from simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

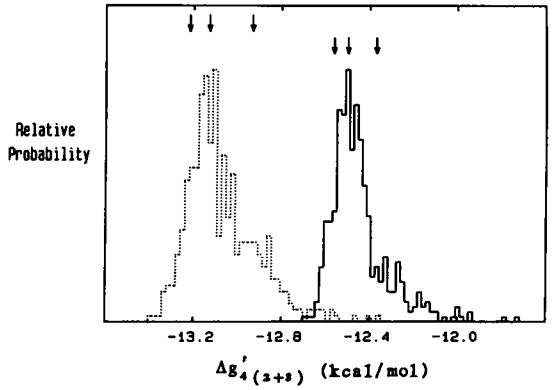

FIGURE 6 Derived sum of the intermediate intrinsic tetramer stepwise oxygen-binding free-energy changes for binding the second and third oxygens to tetramers ($\Delta g'_{4(2+3)}$) from simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

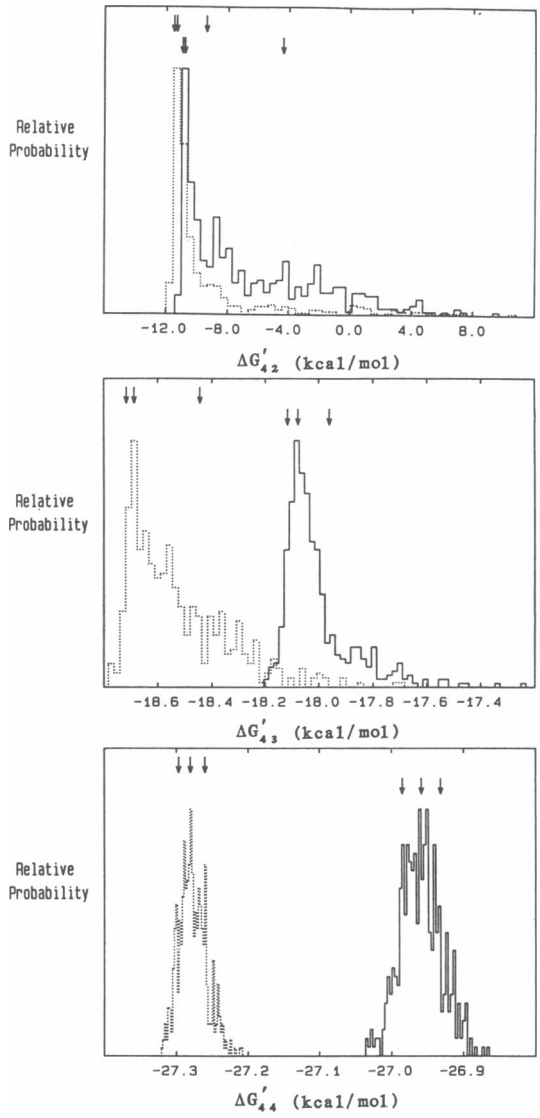

FIGURE 7 Derived intrinsic free energy changes corresponding to the product Adair oxygen binding constants for binding two, three, and four oxygens to unliganded tetramers ($\Delta G'_{4i}$, i = 2, 3, 4) from simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

Given the nature of the probability distributions just described, it is not surprising to find that the probability distributions of the free energy changes corresponding to the intrinsic tetramer product Adair oxygen binding constants ($\Delta G'_{4i}$, see Fig. 7) are relatively narrow for $\Delta G'_{41}$, $\Delta G'_{43}$, and $\Delta G'_{44}$ and quite broad for $\Delta G'_{42}$. The distribution of $\Delta G'_{41}$, $\Delta G'_{43}$, and $\Delta G'_{44}$ range over ~0.1 to 1 kcal/mol (note that $\Delta G'_{41}$ is equivalent to $\Delta g'_{41}$ in Fig. 5). The distribution of $\Delta G'_{42}$, on the other hand, spans over ~20 kcal/mol.

The intrinsic oxygen binding affinites of the various liganded hemoglobin tetramers relative to that of dimers is considered in the graphs presented in Fig. 8. The probability distributions of $\Delta g'_{4j} - \Delta g'_{2i}$ represent the differences between the intrinsic tetramer oxygen-binding free-energy change for binding the jth oxygen to tetramers and that for binding oxygen to dimers. Binding of the first and second oxygens to tetramers is clearly less favorable than binding to dimers as none of the probability distributions of $\Delta g'_{41} - \Delta g'_{2i}$ or $\Delta g'_{42} - \Delta g'_{2i}$ enter into the negative free energy domain. The relative affinity for binding the third oxygen, however, is poorly defined and has considerable probability in both the positive- and negative-free energy domains (positive: 33%, Chu; 65%, Mills; negative: 67%, Chu; 35%, Mills).

The probability distributions derived for $\Delta g'_{44} - \Delta g'_{2i}$ (the quaternary enhancement free energy change) suggest that hemoglobin tetramers exhibit enhanced affinity for binding oxygen to their last available site relative to the affinity exhibited by dimers for binding oxygen to their last available site. The simulations performed on the data of Chu et al. (1984) produce a probability distribution for $\Delta g'_{44} - \Delta g'_{2i}$ that exists entirely in the negative free energy domain with a most probable derived value of −0.56 kcal/mol and 67% confidence limits of −0.73 kcal/mol and −0.48 kcal/mol. Simulations performed on the Mills et al. (1976) data produce a distribution with only 0.4% probability of a positive quaternary enhancement free energy change, the most probable derived value being −0.20 kcal/mol with 67% confidence limits of −0.48 kcal/mol and −0.11 kcal/mol. The range of

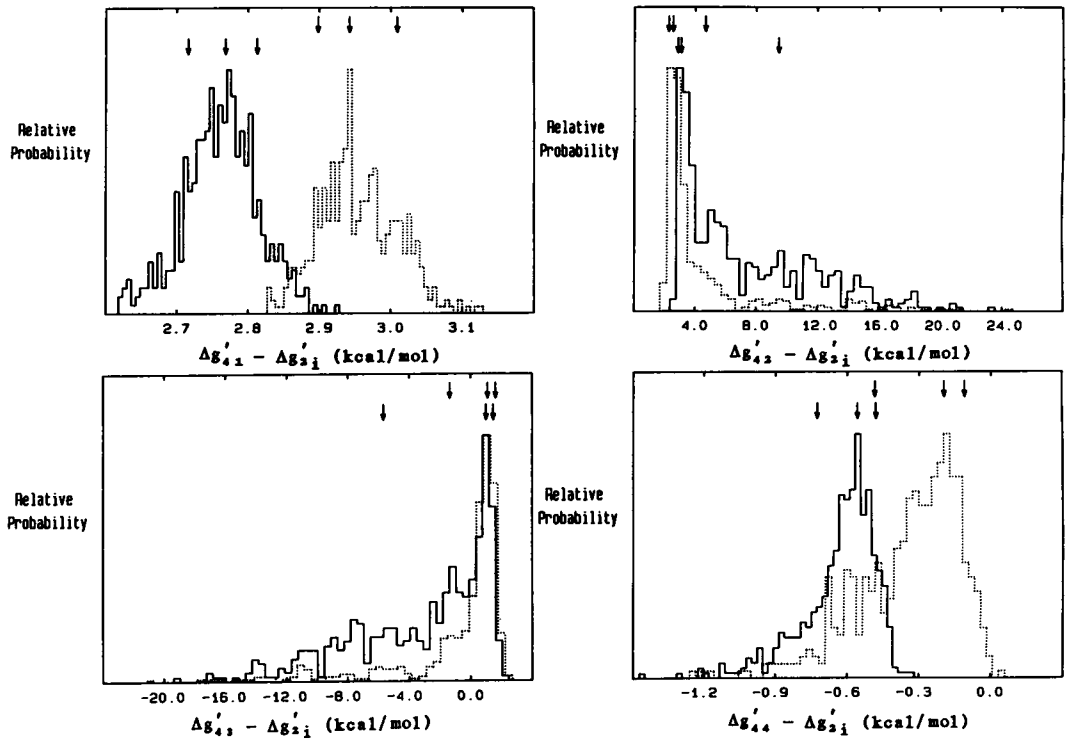

FIGURE 8 Derived differences between the intrinsic tetramer oxygen-binding free-energy changes for forming j-liganded tetramers and the intrinsic dimer oxygen-binding free-energy change ($\Delta g'_{4,j} - \Delta g'_{2,i}, j = 1, 2, 3, 4$) from simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

quaternary enhancement free energy changes consistent with these simulations on both the data of Chu et al. (1984) and Mills et al. (1976) (at 67% confidence) is therefore between −0.11 and −0.73 kcal/mol. This result (*a*) suggests that the quaternary enhancement free energy change exhibited by human hemoglobin, for the conditions under which the original data were acquired, is resolvable to the level of tenths of kilocalories per mole and (*b*) supports the existence of a finite, negative quaternary enhancement free energy change. The two sets of variable protein concentration equilibrium oxygen binding experiments considered here therefore appear to be of sufficient quality to support, with a high degree of confidence, the existence of a finite and negative quaternary enhancement free energy change. We feel that our ability to resolve the quaternary enhancement effect is a direct consequence of utilizing two dimensional oxygen binding data: i.e., variations in both oxygen and hemoglobin concentrations.

The differences in free energy changes presented in Fig. 9 represent the cooperative free energy levels associated with the variously liganded hemoglobin tetramers relative to the unliganded tetramer form (Ackers and Smith, 1987; Smith and Ackers, 1985). These cooperative free energy parameters have particular significance when the functioning of the hemoglobin tetramer is considered in the context of a combinatorial switch model (Ackers and Smith, 1987; Smith and Ackers, 1985; Straume and Johnson, 1988). Such a model considers occupation of particular combinations of tetramer ligand binding sites as the "switching" mechanism responsible for inducing discrete changes of cooperative ligand binding free energy in the tetramer. Consideration of the thermodynamics of hemoglobin function within the framework of the oxygen binding and subunit association linkage scheme employed in the present studies does not permit discrimination among different combinatorial forms of equivalently liganded hemoglobin tetramers (i.e., the two singly liganded forms, the four doubly liganded forms, and the two triply liganded forms). However, even with this limitation prohibiting rigorous interpretation with regard to all possible combinatorial switch models, some important relationships become evident by considering the results presented in Fig. 9.

Approximately 2.72 to 3.01 kcal/mol of cooperative free energy are gained by tetramers upon binding of the first oxygen ($^1\Delta G'_2 - {}^0\Delta G'_2$). Binding of the first three oxygens produces ~6.54 to 7.07 kcal/mol of cooperative

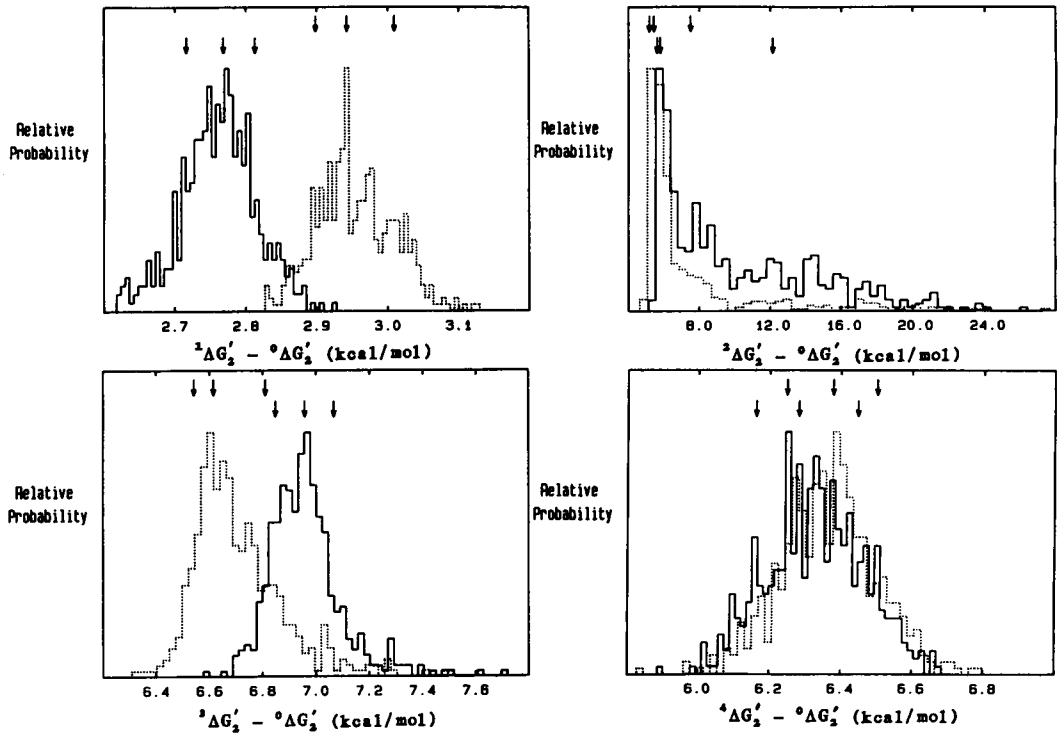

FIGURE 9 Derived differences between the intrinsic dimer to tetramer association free energy changes for forming i-liganded tetramers from appropriately liganded dimers and that for forming unliganded dimers ($^i\Delta G_2' - {}^0\Delta G_2'$, i = 1, 2, 3, 4) from simulations of the data of Chu et al. (1984) (*solid line, lower arrows*) and Mills et al. (1976) (*dotted line, upper arrows*). Arrows correspond to the derived most probable values and lower and upper 67% confidence limits. See Table 1 for a summary of all parameter values.

free energy ($^3\Delta G_2' - {}^0\Delta G_2'$) and fully ligating the hemoglobin tetramer generates ~6.16 to 6.50 kcal/mol of cooperative free energy ($^4\Delta G_2' - {}^0\Delta G_2'$). Each of these three tetramer cooperative free energy levels are relatively narrowly defined (~±0.15 to ±0.27 kcal/mol). Binding two oxygens to hemoglobin tetramers, however, produces between ~5.2 and 12.1 kcal/mol of cooperative free energy ($^2\Delta G_2' - {}^0\Delta G_2'$). The magnitude of the tetramer response to occupation of two of its four oxygen binding sites is therefore poorly defined (Mills et al., 1976; Johnson et al., 1976).

Given the relatively narrow ranges derived (at 67% confidence) for the cooperative free energy levels associated with the binding of one, three, or four oxygens to hemoglobin tetramers and the poorly defined, broad distributions obtained for that associated with binding two oxygens to hemoglobin tetramers, it is apparent that a four state combinatorial switch model is capable of adequately characterizing these two sets of equilibrium oxygen binding data. This conclusion, of course, requires that all equivalently liganded tetramers occupy the same cooperative free energy level. One level (with 0 kcal/mol of cooperative free energy) is required for the unliganded, reference tetramer state. A second level is needed (with 2.72 to 3.01 kcal/mol of cooperative free energy) for the singly liganded tetramer form. The triply liganded tetramer form requires a third level (with 6.54 to 7.07 kcal/mol of cooperative free energy) and a fourth level is necessary to account for the fully liganded tetramer form (with 6.16 to 6.50 kcal/mol of cooperative free energy). Based on the ranges of the distributions presented in Fig. 9, the doubly liganded tetramer form may occupy either the third cooperative energy level (6.54 to 7.07 kcal/mol) or the fourth cooperative energy level (6.16 to 6.50 kcal/mol).

The combinatorial switch configuration corresponding to the doubly liganded tetramer form possessing the third cooperative free energy level (6.54 to 7.07 kcal/mol) previously has been identified as one which provides a satisfactory fit to both the data at Chu et al. and Mills et al. (Straume and Johnson, 1988). Based on the information contained in Fig. 9 and discussed in the previous paragraph, we tested the validity of the combinatorial switch configuration in which doubly liganded tetramers occupy the fourth cooperative free energy level (6.16 to 6.50 kcal/mol). We found that it also produces a statistically valid fit to both the data of Chu et al.. (1984) and Mills et al. (1976). It should be pointed out, however, that

these results do not rule out the possibility of other four state combinatorial switch configurations as accurate descriptions of these data, but merely indicate that four state combinatorial switch models are capable of accurately accounting for the equilibrium oxygen binding data considered here.

The method used in the present work to derive the probability distributions for various free energy change parameters characteristic of hemoglobin behavior provides a mechanism to estimate uncertainties in derived model parameters that requires a minimum of assumptions. All correlations among model parameters are implicitly retained by this technique and the effects of these correlations are contained in the resultant probability distribution functions. In addition, the present application also accounts for uncertainties in the unliganded and fully liganded dimer to tetramer association free energy changes, ${}^{0}\Delta G_2'$ and ${}^{4}\Delta G_2'$. The original reports of Chu et al. (1984) and Mills et al. (1976) did not consider the effects of uncertainties in these parameters when estimating confidence limits for the derived model parameters.

We thank Dr. Gary K. Ackers for supplying us with the original equilibrium oxygen binding data.

This work was supported by National Institutes of Health grants GM28928 and AM22125.

Received for publication 10 August 1988 and in final form 13 February 1989.

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