Analysis of oxygen binding by hemoglobin on the basis of mean intrinsic thermodynamic quantities

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The binding data for oxygenation of human hemoglobin, Hb, at various temperatures and in the absence and presence of 2,3-diphosphoglycerate, DPG, and inositol hexakis phosphate, IHP, were analyzed for extraction of mean intrinsic Gibbs free energy, \( \Delta G^o \), enthalpy, \( \Delta H^o \), and entropy, \( \Delta S^o \), of binding at various partial oxygen pressures. This method of analysis considers all the protein species present such as dimer and tetramer forms which were not considered by Imai et al. (Imai K et al., 1970, Biochim Biophys Acta 200: 189–196), in their analysis which was based on Adair equation. In this regard, the values of Hill equation parameters were estimated with high precision at all points of the binding curve and used for calculation of \( \Delta G^o \), \( \Delta H^o \) and \( \Delta S^o \) were also calculated by analysis of \( \Delta G^o \) values at various temperatures using van’t Hoff equation. The results represent the enthalpic nature of the cooperativity in Hb oxygenation and the compensation effect of intrinsic entropy. The interpretation of results also to be, into account the decrease of the binding affinity of sites for oxygen in the presence of DPG and IHP without any considerable changes in the site–site interaction (extent of cooperativity). In other words, the interactions between bound ligands, organic phosphates and oxygen, are more due to a decreasing binding affinity and not to the reduction of the cooperative interaction between sites. The results also document the more heterotropic effect of IHP compared to DPG.

Keywords: hemoglobin, cooperativity, mean intrinsic Gibbs free energy, linkage phenomena

INTRODUCTION

Hemoglobin, Hb, is the respiratory protein in red blood cells of vertebrates. Its physiological functions are transport of oxygen from lungs to tissues and transfer of carbon dioxide from tissues to lungs. The equilibrium of oxygen binding is influenced by organic phosphates such as 2,3-diphosphoglycerate, DPG, which is present in red blood cells of mammals and inositol hexakis phosphate, IHP, which is related to inositol pentakis phosphate found in red blood cells of birds (Baldvin, 1975). This organic phosphate has heterotropic allosteric effect on oxygenation of hemoglobin. Some hemoglobin properties in this regard are interpreted on the basis of fully liganded and/or unliganded hemoglobin structures, while other its properties are explained on the basis of intermediate structures and alternative oligomeric forms of hemoglobin. These are not directly visible and must be illustrate by a model (Roughton, 1936; Imai & Yonetani, 1974; 1975; Imai, 1979; Yamamoto & Nagaoka 1998; Scalonia et al., 1999; Labergea et al., 2005).

The shape of oxygen equilibrium curves is invariant for temperature changes and this led Wyman (1948) to conclude that the heat of oxygenation is the same for all the hemes, and the cooperative effects are essentially entropic in nature. In contrast Imai and Yonetani (1975) believe that the cooperativity of hemoglobin oxygenation may be due to both enthalpic and entropic origins. Imai and Yonetani analyzed the oxygen-hemoglobin binding data by fitting them to Adair equation (Adair, 1925; Adair et al.,

Abbreviations: DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; IHP, inositol hexakis phosphate.
While the estimation of sequential binding constants is possible by this method, the accuracy of the calculated parameters is strongly dependent on the estimation of the initial values and fitting procedures. For example, Brodersen et al. (1987; 1988) reported 30 different acceptable solutions for the oxygen-Hb system. Thus, there is a significant uncertainty on the interpretations that arise from these fitting data. Moreover, those authors did not consider the presence of the dimer form of hemoglobin in their analyzing method. Besides these sequential binding values, the estimation of mean binding parameters is also possible for this system. While it seems that these mean parameters may be less informative than the sequential ones, their estimation is more precise.

In the present work, the mean intrinsic Gibbs free energy, ΔG, enthalpy, ΔH, and entropy, ΔS, of human hemoglobin oxygenation at various experimental conditions such as temperature, presence and absence of DPG and IHP, were calculated using modified form of a formula which was derived earlier (Bordbar et al., 1997). Some new features of the oxygen-Hb equilibrium such as the enthalpic or entropic origin of cooperativity and the nature of the allostric effects of DPG and IHP were revealed through this new analyzing method.

DATA ANALYSIS

The oxygen-Hb binding data were taken directly from literature (Imai & Yonetani, 1974). These data were determined by an automatic recording method as described elsewhere (Imai et al., 1970). The buffers used were 0.05 M bis-Tris with and without 2 mM DPG and 2 mM IHP, and contained 0.1 M Cl⁻ which was adjusted with sodium chloride. The pH of the buffers was adjusted to 7.4 at the same temperatures as for oxygen equilibrium measurements, i.e. at 10, 15, 20, 25, 30 and 35°C.

The corresponding Hill plots were constructed as the variation of log(Y/(1−Y)) versus logPₐ, where Y and Pₐ are fractional saturation of Hb with oxygen and partial oxygen pressure, respectively. The Hill plots were fitted to a suitable equation using Sigma Plot software (http://www.spss.com/software/science/sigmaplot/, and for more information refer to http://www.spssscience.com/). This fitting equation interpolated the experimental data with high precision and was used for determination of exact values of Hill coefficient, nₜ, and Hill binding constant, Kₜ, at any specified value of Pₒ₂. These parameters were calculated using Eqns. (1) and (2), respectively (Hill, 1910)

\[
Kₜ = \frac{Pₒ₂}{Pₒ₂^*}
\]

where Pₒ₂ is pressure of gas at standard conditions of 1 bar. The unit of Kₜ is torr⁻¹. The fitted Hill plots and the variation of calculated nₜ versus logPₒ₂ at various temperatures and in the absence or presence of DPG and IHP are shown in Figs. 1 and 2, respectively.

The following multiple equilibria must exist in solution:

\[
\begin{align*}
\text{Hb}^0 + O₂ & \rightleftharpoons \text{Hb}^0O₂ \\
\text{Hb}^0O₂ + O₂ & \rightleftharpoons \text{Hb}^0(O₂)₂ \\
\text{Hb}^0(O₂)₂ + O₂ & \rightleftharpoons \text{Hb}^0(O₂)₃ \\
\text{Hb}^0(O₂)₃ + O₂ & \rightleftharpoons \text{Hb}^0(O₂)₄
\end{align*}
\]

\[
\frac{d\ln(Y/(1−Y))}{d\ln(Pₒ₂/Pₒ₂^*)} = nₜ
\]
where $K_{oD}^j$ and $K_{oT}^j$ are stepwise intrinsic binding constants for binding of jth $O_2$ to dimer, Hb$^D$, and tetramer, Hb$^T$, forms of Hb, respectively. The intrinsic binding free energy corresponding to these binding constants should be equal to $\Delta G_{oD}^j = -RT \ln(K_{oD}^j \times P^o)$ and $\Delta G_{oT}^j = -RT \ln(K_{oT}^j \times P^o)$, respectively.

The mean intrinsic Gibbs free energy per mole of monomer at any specified value of $P_{O_2}$, $\Delta G^o$, is defined as:

$$\overline{\Delta G^o} = (1/4) \sum_{j=1}^{d} \sum_{i=1}^{j} x^T_i \Delta G^T_j + (1/2) \sum_{j=1}^{T} \sum_{i=1}^{j} x^D_i \Delta G^D_j$$

(3)

where $x^T_i$ and $x^D_i$ are mole fractions of Hb$^T(O_2)_i$ and Hb$^D(O_2)_i$, respectively. $\Delta G^o$ can be calculated using the following equation:

$$\Delta G^o = -n_H RT \ln(K_H \times P^o) + (1 - n_H) RT \ln(P_{O_2} / P^o)$$

(4)

where R and T are universal gas constant and absolute temperature, respectively. This equation is a modified form of the equation which was previously obtained by Bordbar et al. (1997) and applied for binding of ionic surfactants to globular proteins.

Figure 2. Variation of Hill coefficient, $n_{H}$, versus $\log P_{O_2}$. Oxygenation of Hb in the absence of organic phosphates (A), presence of 2 mM DPG (B), and presence of 2 mM IHP (C), at (●) 10, (○) 15, (■) 20, (□) 25, (▲) 30, and (△) 35 °C. The $n_H$ values at any specified point were calculated by derivation of fitting equation of Hill plots (Fig. 1).

Figure 3. Variation of mean intrinsic Gibbs free energy, $\Delta G^o$, versus $\log P_{O_2}$. Oxygenation of Hb in the absence of organic phosphate (A), presence of 2 mM DPG (B), and presence of 2 mM IHP (C), at (●) 10, (○) 15, (■) 20, (□) 25, (▲) 30, and (△) 35 °C.

The modification of the Bordbar equation, the existence of a single set of binding sites in Hb and the variable nature of $n_H$ and $K_H$ were considered. In order to dimensionless of logarithm arguments, the $K_H$ multiply to $P^o$ in the first term and $P_{O_2}$ divided to $P^o$ in the second term of Eqn. (4).

Figure 4. Variation of mean intrinsic Gibbs free energy $\Delta G^o$, versus $\log P_{O_2}$ for oxygenation of Hb in the absence and presence of DPG and IHP, respectively. The variation of $\Delta G^o$ is also shown in Fig. 4, at 25°C in the ab-
The oxygenation data and curve fitting are presented as the Hill plots in Fig. 1, where the observed points are compared with calculated lines. They show excellent fit along the saturation that was covered by experiments. It is evident that the position of the oxygen equilibrium curve depends on the temperature and the presence of DPG or IHP. It also shows that the lower asymptotes converge, while the upper asymptotes diverge, indicating that the initial steps of hemoglobin oxygenation depend on the temperature less markedly than the final steps.

As seen in Fig. 2, the maximum value of Hill coefficient, \( n_{H,max}' \) also depends on the temperature; it become smaller as the temperature is going up in the absence of allosteric ligands (DPG and IHP) and in the presence of DPG. However, it slightly increases with increasing temperature in the presence of IHP.

The mean intrinsic Gibbs free energy is shifted to the right and to less negative values, with increasing temperature (Fig. 3). This means that increasing the temperature reduces both the intrinsic binding affinity of the sites and the cooperative interactions between them. Figure 4 shows the variation of \( \Delta G^0 \) versus \( \log P_{O_2} \) at 25°C in the absence and presence of DPG and IHP. It is obvious that the effect of DPG and IHP on the binding site affinity is stronger than that on the cooperative interaction between the sites.

The linearity of van’t Hoff plots, \( \Delta G^0/T \) versus \( 1/T \), is shown in Fig. 5. With respect to Eqn. (5) the slope and y-intercept of this plot are equal to the mean intrinsic heat change \( \Delta H^0 \), and the negative value of the mean intrinsic entropy change, \( -\Delta S^0 \) at any specific value of \( P_{O_2} \), respectively. The variation of \( \Delta H^0 \) and \( -\Delta S^0 \) versus \( \log P_{O_2} \) are shown in Fig. 6.

\[
\Delta G^0/T = \Delta H^0/T - \Delta S^0
\]  

**RESULTS AND DISCUSSION**

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\[
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\]  

Figure 5. Variation of \( \Delta G^0/T \) versus \( 1/T \) for oxygenation of Hb:
- (A) in the absence of organic phosphate, (●) \( P_{O_2} = 1 \), (○) \( P_{O_2} = 1.236 \), (■) \( P_{O_2} = 1.528 \), (□) \( P_{O_2} = 1.888 \), (▲) \( P_{O_2} = 2.333 \), (△) \( P_{O_2} = 2.884 \), (▼) \( P_{O_2} = 3.565 \), (▽) \( P_{O_2} = 4.395 \), (○) \( P_{O_2} = 5.445 \), (△) \( P_{O_2} = 6.730 \), (●) \( P_{O_2} = 8.318 \) torr,
- (B) in the presence of 2 mM DPG, (●) \( P_{O_2} = 2.698 \), (○) \( P_{O_2} = 3.873 \), (■) \( P_{O_2} = 5.483 \), (□) \( P_{O_2} = 7.047 \), (▲) \( P_{O_2} = 10.000 \), (△) \( P_{O_2} = 13.274 \), (▼) \( P_{O_2} = 18.535 \), (▽) \( P_{O_2} = 25.882 \), (○) \( P_{O_2} = 36.141 \), (△) \( P_{O_2} = 59.293 \), (●) \( P_{O_2} = 83.946 \), and (□) in the presence of 2 mM IHP, (●) \( P_{O_2} = 10.7 \), (○) \( P_{O_2} = 14.8 \), (▲) \( P_{O_2} = 21.0 \), (▼) \( P_{O_2} = 29.5 \), (■) \( P_{O_2} = 41.4 \), (□) \( P_{O_2} = 58.1 \), (▲) \( P_{O_2} = 81.5 \), (△) \( P_{O_2} = 114.3 \), (●) \( P_{O_2} = 160.3 \), (○) \( P_{O_2} = 225.4 \), (▲) \( P_{O_2} = 315.5 \) torr.

Figure 6. Variation of \( \Delta H^0 \) (●, ▲, and △) and \( \overline{T\Delta S^0} \) (○, □, and △) versus \( P_{O_2} \).

Oxygenation of Hb, in the absence of organic phosphate (circles), in the presence of 2 mM DPG (rectangles) and in the presence of 2 mM IHP (triangles).
Oxygen binding by hemoglobin on the basis of mean intrinsic thermodynamic quantities

An analysis of oxygen binding data on the basis of their fitting to Adair equation has been done previously and most of the interpretations presented are based on the results of this method of analysis (Imai & Yonetani, 1974; 1975; Imai, 1979). The major shortcomings of this method are the limitations of this equation in covering all data; the calculated results are of low precision and accuracy, and dimer contribution is neglected. In this work, we fit the binding data to a suitable equation with high precision that subsequently allowed us to calculate Hill binding parameters with a thermodynamic basis through Eqn. (4). Thus, our results are more precisely analyzed for extraction of mean intrinsic thermodynamic quantities. We believe that this new analysis method can be also used for any binding system and its importance is going up with an increasing number of binding sites which creates more uncertainty in the estimated values of Adair binding constants.

The entropic origin of the cooperativity of hemoglobin oxygenation was introduced by Wyman (1948), whereas Imai and Yonetani (1975) believe that the cooperativity may have both enthalpic and entropic origins. With respect to the negative values of mean intrinsic enthalpy and entropy of hemoglobin oxygenation in the absence and presence of organic phosphates (Fig. 6), enthalpy causes a decrease of the mean intrinsic Gibbs free energy, while entropy increases it. Therefore, we propose that the cooperativity of hemoglobin oxygenation has enthalpic origin and entropic compensation. The interpretation of the results shows a decrease of the site binding affinity in the presence of organic phosphate without any considerable changes in the amount of site–site interaction (extent of cooperativity). On the other hand, the heterotropic linkage phenomenon in simultaneous binding of organic phosphate and oxygen to Hb is more due to decreasing of binding affinity of sites and less due to reducing of cooperative interaction between sites.

The sensitivity of this approach to the uncertainty in Hill parameters was also tested and the result is shown in Fig. 7. This figure shows the variation of calculated $\Delta G^\circ$ versus $\log P_{O_2}$ at $10^\circ C$ and in the absence of DPG and IHP, by considering 5% relative error in estimated $n_H$ and $K_H$ values. The relative error in $\Delta G^\circ$ was less than 5% in all points.
Moreover, the trend of variation of $\Delta G^0$ that has an essential role in our interpretation, shows little dependency on such errors. This shows that the proposed method is more reliable compared to Adair equation fitting.

Several blood diseases are due to structural and genetic defects of hemoglobin (Chinchanga et al., 2005; Nishino et al., 2006; Shmukler et al., 2000; Stathopulos, 2003; Steiper et al., 2006). These diseases could affect hemoglobin oxygenation activity. Our approach can be used to investigate the effect of these structural changes on function of Hb. The obtained results may lead us to designing new drugs for therapy of these diseases.
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