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Enthalpy change of the allosteric transition in human haemoglobin A

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Abstract

Oxygen equilibria of haemoglobin were analysed using binding isotherms to obtain the intrinsic oxygen association constants to the haem moities of haemoglobin. Two sets of binding sites in haemoglobin were identified, which were ascribed to the *R* and *T* forms of the haems. The average intrinsic association constants, determined as a function of temperature, gave a heat of oxygenation of 76 ± 4 kJ mol^{-1} for the haemoglobin tetramer. A microcalorimetrically determined heat of dissociation of oxyhaemoglobin by dithionate was -267 ± 10 kJ mol^{-1} (tetramer). From these results, the heat of allostery of -343 ± 14 kJ mol^{-1} of the haemoglobin tetramer was obtained, yielding an allosteric energy per mole of salt-bridge of -42 ± 4 kJ mol^{-1}. This results suggest strongly that the salt-bridges may be hydrogen bonds. © 1997 Elsevier Science B.V.

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1. Introduction

The quaternary structural changes that accompany cooperative ligand binding by haemoglobin have been analyzed extensively by Perutz [1] and Baldwin and Chothia [2]. Although both analyses are based on the existence of two thermodynamically stable quaternary structures, it was recently shown that haemoglobin crystals grown at lower salt concentrations, identical with physiological conditions, yield a third quaternary structure termed *Y* [3,4] or R_2 [5]. However, computational experiments showed that linear interpolation from *T* (tense) to R_2 passed through *R* (relax); that is to say that, R_2 is a more relaxed form of *R* [6].

Of greater difficulty, however, is the direct measurement of the energy accompanying the allosteric transition. Recent reports by Ackers et al. [7], based on measurement of oxygen equilibrium curves at various haemoglobin concentrations, gave allosteric free energies of the order of 25 kJ mol^{-1} for various ligation species of the cyano-methaemoglobin. The enthalpy change quoted for the allosteric process of haemoglobin of the order of 42–50 kJ mol^{-1} [1], is even less satisfactory. As haemoglobin continues to play a major role in the progress towards understanding the detailed structural and energetic basis of allosteric regulation of enzymes in general, this study reports the contribution to the thermodynamics of the allosteric process.

Many methods of analysis of haem–ligand binding have been described for the oxygenation equilibria of many types of haemoglobin [8]. Recently, a theory

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was proposed for the spontaneous self-assembly of surfactant molecules onto a solid substrate [9]. This method should apply equally well to homotropic ligand binding by haemoglobin. Since haemoglobin is comparatively more massive (MW 64 500) when compared with oxygen (MW 32), the rate of diffusion is of the order of 2% of that of haemoglobin. The haemoglobin molecule may be, therefore, assumed to be more stationary while oxygen is mobile. The advantage of the recent method [9] of analysis over existing ones is that it is capable of yielding information directly on the intrinsic binding constant of a ligand to a haemoglobin haem; and it is very sensitive to small changes in the affinities of the binding sites.

In this paper, the oxygen binding constant to human haemoglobin A is measured using both the tonometer and continuous oxygenation process. We have also obtained the enthalpy change accompanying the oxygen process using sensitive isothermal titration microcalorimetry.

2. The theory

Let the reaction between haemoglobin and its homotropic ligands, L represented by,

$$\begin{aligned} &(\mathrm{Hb})_0 + L \rightleftarrows (\mathrm{Hb})_1 \\ &(\mathrm{Hb})_1 + L \rightleftarrows (\mathrm{Hb})_2 \\ &\qquad\vdots \\ &(\mathrm{Hb})_{i-1} + L \rightleftarrows (\mathrm{Hb})_i \end{aligned} \tag{1}$$

Suppose further that $(\mathrm{Hb})_0$, $(\mathrm{Hb})_1$,. . .,$(\mathrm{Hb})_i$,. . ., represent concentrations of haemoglobin molecules in which, 0,1,. . .,i,. . . haems are liganded with oxygen. Since, at equilibrium, $(\mathrm{Hb})_0$ must remain constant, the rate of desorption of an oxygen molecule from a liganded haem on the protein and, thus,

$$k_+ c(\mathrm{Hb})_0 = k_-(\mathrm{Hb})_1 \mathrm{e}^{-E_1/RT} \tag{2}$$

where c is the concentration (in mol dm^{-3}) of oxygen in haemoglobin solution, k_+ and k_- are the rate constants of adsorption and desorption, respectively, and E_1 is the heat of adsorption of oxygen to a haem. This is essentially Langmuir's formulation for unimolecular adsorption [10] and involves the assumption that k_+, k_- and E_1 are independent of the number of adsorbed molecules already present on the absorbent.

Furthermore, let the rate of adsorption of an additional oxygen molecule onto a haemoglobin with i liganded haems be k_+ and the rate of desorption be ik_-, since there are i ways in which the oxygen molecule can leave. Thus, in a dynamic equilibria,

$$k_+ c(\mathrm{Hb})_{i-1} = ik_-(\mathrm{Hb})_i \mathrm{e}^{-E_i/RT} \tag{3}$$

Consequently, the number of liganded haems per haemoglobin tetramer obeys the Poisson distributiion and the free energy of adsorption falls off with increase in the number of oxygen molecules already present on the haemoglobin tetramer. We may make the simplifying assumption that the intrinsic heat of adsorption to the haems are equal, that is $E_1 = E_2 = \ldots = E_i = E_a$, and obtain from Eqs. (2) and (3) the total concentration of haemoglobins with i liganded haems,

$$(\mathrm{Hb})_i = (\mathrm{Hb})_0 \frac{\left(kc\mathrm{e}^{E_a/RT}\right)^i}{i!} \tag{4}$$

where the intrinsic equilibrium constant $k = k_+/k_-$. If we define an observed equilibrium constant $k_0 = k\exp(E_a/RT)$, then Eq. (4) becomes,

$$(\mathrm{Hb})_i = (\mathrm{Hb})_0 \frac{(k_0 c)^i}{i!} \tag{5}$$

The total number of haems in tetramer available for binding oxygen is given by,

$$(\mathrm{Hb})_T \equiv \sum_{i=0}^{\infty} (\mathrm{Hb})_i = (\mathrm{Hb})_0 \sum_{i=0}^{\infty} \frac{(k_0 c)^i}{i!} = (\mathrm{Hb})_0 \mathrm{e}^{k_0 c} \tag{6}$$

The summation has been extended to infinity so that the final equation is valid for haem proteins like erythrocruorins with over 80 haems. The fraction of haemoglobin with i liganded haems is given by,

$$\theta_i \equiv \frac{(\mathrm{HB})_i}{(\mathrm{Hb})_T} = \frac{(k_0 c)^i / i!}{\mathrm{e}^{k_0 c}} \tag{7}$$

thus, θ_i is fractional saturation. Application of Sterling's approximation to Eq. (7) in the logarithmic form, and rearrangement, gives

$$\ln \theta_i - i \ln c = b - k_0 c \tag{8}$$

where $b = i(\ln k_0/i + 1)$ is a constant for a given value of i. Eq. (8) represents adsorption of oxygen from solution onto haemoglobin at equilibrium. We

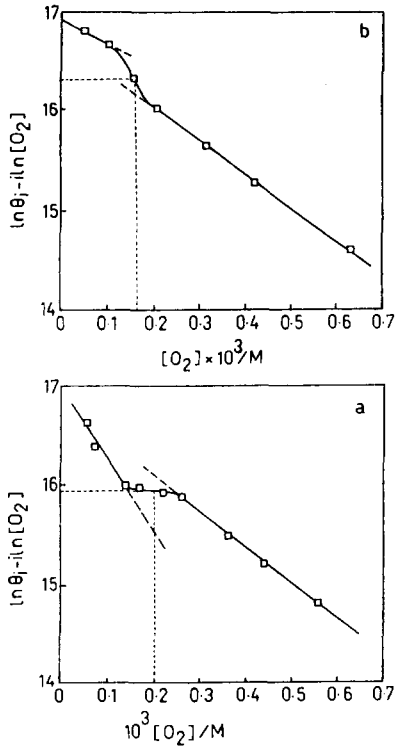

Fig. 1. $\ln \theta_i - i \ln[O_2]$ vs. $[O_2]$ mol dm^{-3}. (a) – Deoxygenation of human haemoglobin A; [HbA] = 0.125 mM (haem), $T = 25 \pm 1$°C, pH = 7.2, Bis-Tris-HCl buffer ($I = 0.05$ M). (b) – Oxygenation of human haemoglobin A (using tonometer); [Hb] = 1.68×10^{-5} mol dm^{-3} (haem), $T = 25 \pm 1$°C Tris-HCl buffer ($I = 0.01$ M) pH = 7.2. (It may be noted that oxygenation curve is a mirror image of deoxygenation curve as expected.)

employed two different methods in obtaining haemoglobin oxygen equilibrium. Points were determined while deoxygenating progressed using the continuous procedure; and the tonometer was used to obtain experimental points for the oxygenation equilibrium. Plots of $\ln \theta i - i \ln c$ against c (mol dm^{-3}) are shown in Fig. 1. The slopes represent the observed intrinsic binding constants for two sets of haems with different affinities for oxygen.

3. Experimental

3.1. Materials

Haemoglobin was prepared by the method of William and Tsay [11]. Concentration of haemoglobin solution was determined by the Austine and Drabkin method [12]; and the haemoglobin concentration used in these experiments, unless otherwise stated, was 1.68×10^{-5} (haem). Tris-HCl buffer (Sigma), pH = 7.2 and I = 0.10 M was prepared by dissolving appropriate amounts of Tris in double-distilled water and adding an appropriate volume of HCl. The ionic strength was made up with weighed quantities of NaCl. A spectrophotometer (Shimadzu model UV-3100) was used with accuracy of 0.001 for absorption measurement. A gyrotor shaker: (New Brunswic Scientific model G-76) with speed at point 3, was used for shaking at constant temperature. Dithionate (Merck) solution was prepared by direct addition of dithionate into the aforementioned de-aerated Tris-HCl buffer.

3.2. Methods

3.2.1. Oxygenation of haemoglobin

Oxygenation was carried out by a tonometric method as described by Rossi-Fanilli and Antonini [13]. The only difference is that a syringe was used for evacuation of the haemoglobin solution in the tonometer. Fractional saturation of haemoglobin was achieved spectrophotometerically using the absorption peak at 580 nm. The equation, $\theta_i = (A_0 - A_s)/(A_0 - A_d)$, where A_0 is the optical density of haemoglobin solution in fully oxygenated form, A_s the absorption at a given partial pressure of oxygen, and A_d the optical density of a completely deoxygenated haemoglobin, was used to calculate θ_i. Eight experimental points were determined at each temperature.

3.2.2. Deoxygenation curve

The procedure used for determining deoxygenation is a modified form of the continuous deoxygenation process reported by Imai et al. [14]. Deoxygenation was carried out in a separate degassing vessel which permits a fine control of the rate of deoxygenation to match the response time of the Clark oxygen electrode type E 5040. This reduced the error that attends the determination of oxygen tension by this method. A plot of the fractional saturation against log P_{O_2} was found to be symmetrical. The deoxygenation experiment was carried out on haemoglobin solutions

(0.125 mM) in Bis-Tris-HCl buffer ($I = 0.05$ M) at pH 7.2 at 300 K.

3.2.3. Calorimetric measurement

The calorimetric experiments were carried out with a four-channel commercial isothermal microcalorimetric system (thermal activity monitor 2277, Thermometric, Sweden). Each channel is a twin heat-conduction calorimeter, where the heat-flow sensor is a semiconductoring thermopile (multijunction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. Dithionate solution (2 mM) was injected by a Hamilton syringe into the calorimeter stirred titration vessel which contained 2 cm^3 haemoglobin solution (2.50 μM), including Tris-HCl buffer (0.10 M), pH = 7.20. A thin (0.15 mm i.d.) stainless steel hypodermic needle, permanently fixed to the syringe, reached directly into the calorimeter vessel. The injection of dithionate into the perfusion vessel was repeated five times, and each injection included 0.025 cm^3 of dithionate solution. The calorimetric signal (with accuracy of 0.1 μw) was measured by a digital voltmeter, part of a computerized recording system. The enthalpy change for each injection was calculated by a "Digitam" computer program. The enthalpy of dilution of the dithionate solutions were measured as described above, except that haemoglobin was excluded. The enthalpy of dilution of the dithionate in the absence of protein was subtracted from the enthalpy in the presence of haemoglobin. The enthalpy of dilution of protein is negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

All experiments were repeated twice and the experimental data were grouped together.

4. Results and discussion

Plots of the left hand side of Eq. (8) against the concentration of oxygen in haemoglobin solution are shown in Fig. 1(a) and (b). The concentration of oxygen was calculated from the relation,

$$[O_2] = \alpha P_{O_2}(\text{mm/Hg}) \quad (9)$$

where α is a proportionality constant derivable from the experimental conditions and the ideal gas equation. Many values of i from one to four were tried, none produced straight lines except the value $i = 2$. This suggests two classes of binding sites. As reported previously [9], this is one further advantage of using the present mode of analysis. It is easy to observe a transition from a low affinity region, in the case of oxygenation, to a higher affinity region. In the deoxygenation plot (Fig. 1(a)), the transition takes place from a low dissociation constant to a high dissociation constant. The midpoint of the transition, in both cases, Fig. 1(a) and (b) approximates 30% oxygenation. It can be obtained from the coordinates of the midpoint of the transition. This may be interpreted to mean that the switch-over point is after unloading of about three molecules of oxygen or after the uptake of one oxygen molecule in the deoxygenated state. For the first time these results demonstrate, in a practical way, all allosteric transition from the deoxy (T) to the fully oxy (R_2 or Y) state of haemoglobin. The values of free-energy change given by $\Delta G = -RT \ln k_0$ were calculated from the two slopes to give low affinity form (T-state) ΔG of -19.9 ± 0.2 kJ mol^{-1} (haem), and high affinity form (R_2 or Y-state) $\Delta G = -21.6 \pm 0.4$ kJ mol^{-1} (haem). The average free energy change -20.8 ± 0.3 kJ mol^{-1} (haem) was obtained from the two values.

Fig. 2. shows a plot of log k_0 vs. $1/T$. From the slope of the straight line in Fig. 2 (with a correlation coeffi-

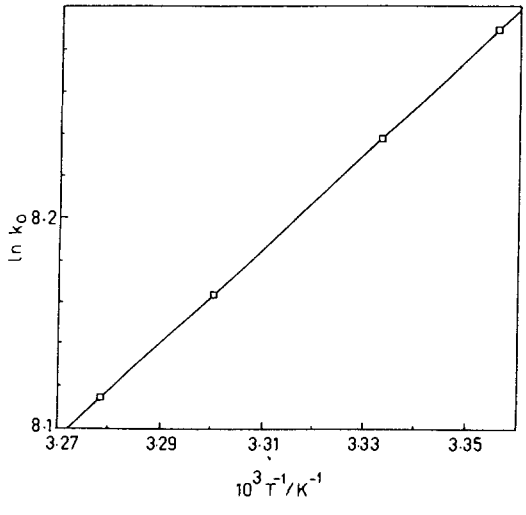

Fig. 2. Influence of temperature on the oxygenation equilibrium of human haemoglobin A. [Hb] = 1.68×10^{-5} M (haem). Tris-HCl Buffer ($I = 0.1$ M) pH = 7.2.

cient of 0.99), a ΔH value of -18.9 ± 2 kJ (haem) was obtained; which may be compared with the value obtained by Hill and Wolvekamp [15] of 41.8 kJ mol^{-1} (oxygen) or 10 kcal. This value was obtained from the reciprocal relationship, $\log K = -\log p_{50}$. The quantity p_{50} represents 50% oxygenation or an average of two liganded haems per tetrameric molecule. By definition, k_0 is the observed intrinsic binding constant to a haem. Thus, the K value used for the calculation of ΔH by Hill and Wolvekamp may be related to k_0 by the equation, $K = (k_0)^2$.

The value of ΔH reported by Hill and Wolvekamp is, therefore, twice the value obtained here. In order to probe the allosteric phenomenon observed in Fig. 1 further, we proposed the following deoxyganation scheme:

$$Hb(O_2)_n \rightleftarrows Hb + nO_2 \quad \Delta H(\text{deoxy}) \qquad \text{(i)}$$

$$Hb(R_2) \rightleftarrows Hb(T) \quad \Delta H(\text{allostery}) \qquad \text{(ii)}$$

in which the heat of deoxygenation accompanying these processes was measured by a sensitive isothermal microcalorimetry. Deoxygenation was carried out with dithionate reaction with oxygen according to equation,

$$S_2O_4^{2-} + O_2 + 2H_2O \rightarrow 2HSO_3^- + H_2O_2 \quad \Delta H_{(\text{dith})} \qquad \text{(iii)}$$

Side effects are known to attend deoxygenation by the use of dithionate [16]. By a careful balance between the concentration of dithionate and haemolgobin, the problems of oxidation of amino-acid residues by hydrogen peroxide and other similar problems were averted.

As the partial pressure of oxygen in haemoglobin solution dropped, the haems simultaneously released their oxygen and the $T \rightarrow R_2(Y)$ transition occurred. The enthalpy change measured with the calorimeter is a composite of the processes in Eqs. (i),(ii) and (iii) above.

The heat of dilution obtained from a dilution experiment, carried out without protein, was subtracted from the heat obtained in the presence of protein. The contribution from reaction (iii) above was thereby eliminated. The total enthalpy change for reactions (i) and (ii), above, ΔH_{tot} has been plotted as a function of total dithionate concentration in Fig. 3. The shape

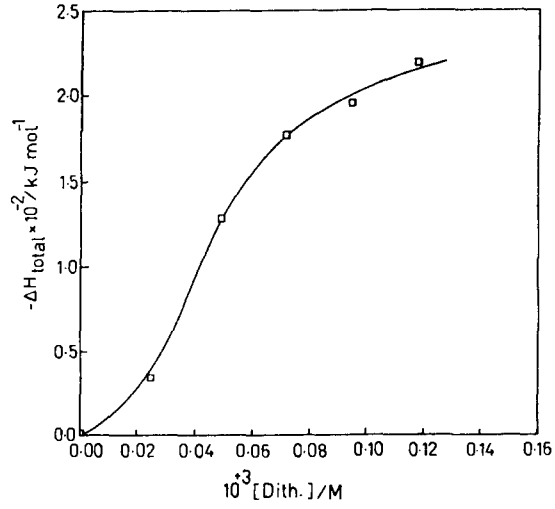

Fig. 3. Enthalpy change accompanying deoxygenation of human haemolgobin A as a function of dithionate concentration. $T = 27 \pm 0.005$°C. Tris-HCl Buffer ($I = 0.10$ M), pH 7.2.

of the curve is sigmoid as expected. Extrapolation of the total enthalpy change, accompanying reactions (i) and (ii), to infinite dithionate concentration gave the infinity enthalpy change (ΔH_∞) of -267 ± 10 kJ mol^{-1} (tetramer). From the foregoing considerations, we can write,

$$\Delta H_\infty = \Delta_{(\text{allostery})} - \Delta H_{(\text{deoxy})} \qquad (10)$$

The negative sign of $\Delta H_{(\text{deoxy})}$ arises from a consideration that the process involves bond breaking while transition from R to T involves bond formation. Given the heat of oxygenation of -76 ± 4 kJ mol^{-1} (tetramer), substitution of the foregoing values into Eq. (10) gave enthalpy change accompanying allosteric effect of -343 ± 14 kJ mol^{-1} (tetramer); equivalent to a heat change of -5 J g^{-1} protein.

From Perutz [1] and Baldwin and Chothia [2], it has been shown that eight salt-bridges were broken in the transition from deoxy (T) to oxy (R_2) state. Assuming that the salt-bridges are chemically equivalent, we may ascribe an allosteric enthalpy change of -42 ± 2 kJ mol^{-1} to each salt-bridge. This is about 10 ± 1 kcal mol^{-1}. This value is reasonable when compared with hydrogen-bonding energy of 6.5 kcal mol^{-1} [17]. The difference in the magnitude of these values may arise from enthalpy entropy compensation resulting from sundry new contacts coupled with new groups free to rotate in aqueous

medium which accompany the allosteric transition [9]. These results, therefore, seem to suggest that salt-bridges may, in fact, be hydrogen-bonds. To the best of our knowledge, enthalpy of allosteric transition has never been previously measured directly.

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