CALORIMETRIC MEASUREMENT OF STEPWISE ENTHALPY CHANGES FOR THE BINDING OF FOUR OXYGENS TO ADULT HUMAN HEMOGLOBIN

L. Noll, B. G. Barisas and S. J. Gill
Department of Chemistry
University of Colorado
Boulder, Colorado 80302

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SUMMARY

The successive enthalpy changes for the four steps of oxygen binding by diphosphoglycerate-free adult human hemoglobin have been measured by direct calorimetry at pH 7.4 and 6°. Average results in kcal/(mole O_2) are: $\Delta H_1 = -25.1 \pm 2.8$; $\Delta H_2 = -12.6 \pm 3.0$, $\Delta H_3 = -12.5 \pm 3.0$, and $\Delta H_4 = -10.1 \pm 1.4$. These results imply a substantial temperature dependence for the cooperativity of O_2 binding by the protein and generally resemble the van't Hoff results by Roughton et al. [Roy. Soc. of London Proc., B 144, 29 (1955)] for sheep hemoglobin at pH 9.1 and a temperature range of 2° to 19°.

INTRODUCTION

The determination of the enthalpy changes ΔH_i for the various stages (i=1 to 4) of ligand binding to hemoglobin has proven extremely difficult. This is because the van't Hoff techniques heretofore employed require extremely precise determinations of the ligand saturation curve at both high and low fractional extents of saturation.

Roughton (1) pointed out that significant differences in ΔH_i may be compatible with the known general temperature invariance of the shape in the middle region of the ligand saturation curve. Wyman (2), however, cited this general temperature invariance as confirming four equal ΔH's. Experiments by Roughton et al. (3) indicated considerable differences in the four van't Hoff enthalpies of reaction using sheep hemoglobin at pH 9.1 in a borate buffer [ΔH_i(i=1 to 4) = -15.7±0.8, -11.4±2.5, -7.8±3.7, -8.7±3.3 kcal/(mole O_2),

respectively]. Later results by Wyman (4) and by Amiconi et al. (5) are, on the other hand, claimed to support uniform enthalpy changes for the four ligand-binding steps. With a view toward resolving these apparent conflicts, we have used direct calorimetry to determine the four successive heats of reaction of oxygen with diphosphoglycerate-free adult human hemoglobin.

METHODS

Adult human hemoglobin was prepared at frequent intervals from freshly-drawn blood using the method of Benesch et al. (6). The hemoglobin concentration was in the range of 2-3 mM heme in a sodium maleate buffer of pH 7.4 and ionic strength 0.3. The maleic acid buffer was used because it has a near zero enthalpy of ionization. An ionic strength of 0.3 was chosen in order to simulate conditions under which the Bohr effect was studied by Antonini et al. (7). Hemoglobin was deoxygenated by rotating the sample in a long tube under reagent grade argon. The progress of deoxygenation was monitored spectrally. The calorimeter was a sensitive dynamic instrument described in detail by Rudolph et al. (8) with an improved stainless steel tube gas handling system. These runs were performed at 5.4°-6.4° in order to minimize methemoglobin formation. The heat evolution and oxygen uptake were followed simultaneously from the beginning to the end of an experiment which took approximately 2 hours.

According to the model of Adair (9) the equilibrium of hemoglobin with oxygen can be represented in terms of four reversible reactions of the form

$$Hb(O_2)_{i-1}(aq)+O_2(g)=Hb(O_2)_i(aq),\ i=1,2,3,4 \qquad (1)$$

Each reaction is characterized by an equilibrium constant K_i and an enthalpy change ΔH_i. When $\bar{n}$ moles of O_2 react with

one mole of deoxyhemoglobin, the heat q absorbed is then given by

$$q=\alpha_1(\Delta H_1)+\alpha_2(\Delta H_1+\Delta H_2)+\alpha_3(\Delta H_1+\Delta H_2+\Delta H_3)+\alpha_4(\Delta H_1+\Delta H_2+\Delta H_3+\Delta H_4) \quad (2)$$

where $\alpha_1 \ldots . \alpha_4$ are the fractions of Hb molecules binding 1 to 4 ligands respectively. Differentiation with respect to $\bar{n}$ gives

$$\frac{dq}{d\bar{n}} = \Delta H_1(\alpha_1'+\alpha_2'+\alpha_3'+\alpha_4')+\Delta H_2(\alpha_2'+\alpha_3'+\alpha_4')+\Delta H_3(\alpha_3'+\alpha_4')+\Delta H_4\alpha_4', \quad (3)$$

where $\alpha_i' = d\alpha_i/d\bar{n}$. The calorimeter gives values of $dq/d\bar{n}$ as a function of $\bar{n}$. Equation (3) thus permits evaluation of the successive enthalpy changes, provided methods are available for the calculation of the $d\alpha_i/d\bar{n}$ factors.

For small values of $\bar{n}$ only the first term of equation (3) is important, and for values of $\bar{n}$ approaching $\bar{n} = 4$ only the last term is significant. Thus

$$\lim_{\bar{n}\to 0} \frac{dq}{d\bar{n}} = \Delta H_1 \quad \text{and} \quad \lim_{\bar{n}\to 4} \frac{dq}{d\bar{n}} = \Delta H_4 \quad (4)$$

The measurement of the overall heat of the reaction gives the total enthalpy $(\Delta H_1+\Delta H_2+\Delta H_3+\Delta H_4)$ for the reaction with four moles of O_2. Thus in principle calorimetric measurements permit direct determination of ΔH_1, ΔH_4 and $\Delta H_2+\Delta H_3$. The resolution of $\Delta H_2+\Delta H_3$ requires the use of additional information contained in the equilibrium constants K_i. This procedure will be described in detail in a separate publication.

Approximate values of ΔH_i were used to correct the K_i values from 25°C to the temperature of the particular experiment assuming the values of ΔH_i are independent of temperature. This set of K_i values was then used to calculate the various derivative coefficients of equation (3) as a function of $\bar{n}$. Since ΔH_t was determined directly from calorimetric data, we ascribed to it high precision and used the identity

$$\Delta H_2 = \Delta H_T - \Delta H_1 - \Delta H_3 - \Delta H_4 \quad (5)$$

Table I: Thermodynamic Parameters for Oxygen Binding to Diphosphoglycerate-free Adult Human Hemoglobin at 25°.

	1st oxygen	2nd oxygen	3rd oxygen	4th oxygen
ΔH (kcal/mole)[a]	-25.1±2.8[e]	-12.6±3.0	-12.5±3.0	-10.1±1.4
K (mm^{-1})[b]	.32	.44	.50	1.09
ΔG (kcal/mole)[c]	-3.25	-3.45	-3.52	-3.98
ΔS (cal/deg-mole)[d]	-73±9	-31±10	-30±10	-21±5

[a]Determined by direct gas-liquid microcalorimetry at 6°, assuming the temperature dependence of the enthalpies to be zero.
[b]From data of Tyuma et al. (1971).
[c]The standard state for O_2 is taken as 1 atm.
[d]Calculated from enthalpies and free energies.
[e]See text for discussion of error estimates.

to eliminate one adjustable parameter from equation (3). A least-squares fit was then made with the experimental data, $\frac{dq}{d\bar{n}}$ given for various values of $\bar{n}$, to determine the parameters ΔH_1, ΔH_3 and ΔH_4. The value of ΔH_2 was calculated by equation (5). The ΔH_i values obtained were then used to provide corrected K_i's and the process repeated until a convergent result was obtained.

Two small corrections were applied to the raw calorimeter data. First, heat evolution due to a slight amount of methemoglobin formation was corrected for by assuming the rate of this reaction to be proportional to the degree of oxygenation of the sample. Second, heat effects due to solution of unreacted oxygen were estimated assuming a value of -3.8 kcal/mole for the ΔH of solution of O_2 at 6°. This correction was applied to the final few points only where it is greater than 1% of the per-point heat evolution. Of the five runs made, one involved Hb from a donor different than that for the other four. Two of the

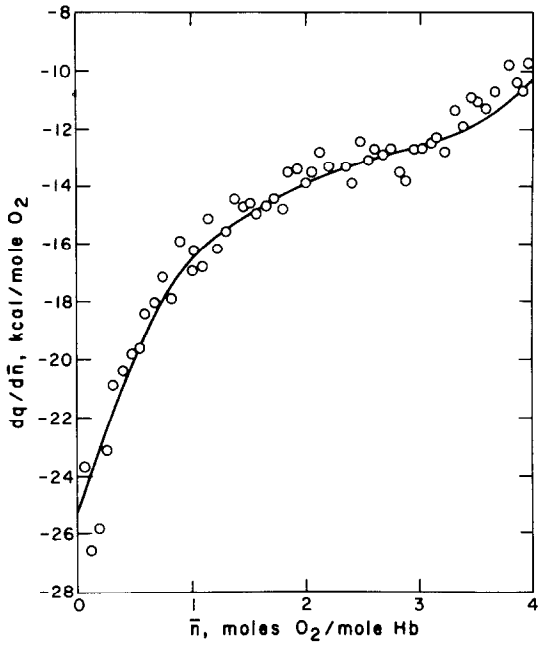

Figure 1: The differential heat $dq/d\bar{n}$ of oxygen binding to diphosphoglycerate-free adult hemoglobin as a function of the average number $\bar{n}$ of ligands bound per mole of protein. Temperature 6°; pH 7.4. The points are the data from a representative calorimeter run, while the smooth curve is calculated from the parameters in Table I. The left intercept of the curve is ΔH_1 while the right intercept is ΔH_4.

runs were made on a single sample which, after the first run, was deoxygenated and replaced in the calorimeter. All five runs gave similar results.

RESULTS AND DISCUSSION

The average of our five runs enables us to present the data in Table I. The error limits given for ΔH_i are ± two standard deviations of the ΔH_i from the various runs, while those given for ΔS_i assume the above uncertainty in ΔH_i and none in ΔG_i. How well these results fit the raw data from one calorimeter run is shown in Figure 1.

From these results we draw the following three conclusions. First, the ΔH's are <u>not</u> uniform, but rather reminiscent of Roughton's work (3) on sheep hemoglobin at pH 9.1. This is

apparent from Figure 1 which clearly shows that the differential heat of oxygenation varies with extent of saturation. Second, the difference in ΔH_1 and ΔS_1 compared to ΔH_4 and ΔS_4 implies that a large structural change has occurred during the oxygenation of stripped hemoglobin. This is consistent with the results of Perutz (11) who showed that the quaternary structure of the hemoglobin molecule goes changes a tense (T) state to a relaxed (R) state when ligands are bound. The similarity in values of the second, third, and fourth steps is surprising and warrants more detailed investigation before drawing further conclusions. Third, at 6°, the reaction is markedly less cooperative than at 25°. This is seen both in the calculated binding constants at the two temperatures and in plots of the α_i vs $\bar{n}$. This reduction in cooperativity is the probable reason we have been able to separate the ΔH's at 6°.

Work is in progress in the analysis of the ΔH_i values in view of various models proposed for the cooperative reaction.

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