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Calorimetric Determination of the Heat of Oxygenation of Human Hemoglobin as a Function of pH and the Extent of Reaction†

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ABSTRACT: Oxygenation of human hemoglobin has been studied by calorimetry at various pH values and buffering conditions. The intrinsic heat of oxygenation was found to be essentially independent of pH in the region of the alkaline Bohr effect, between pH 7.5 and 9.5. A value of -14.1 ± 0.4 kcal/mol of heme was obtained for the intrinsic enthalpy pertaining to total oxygenation in distilled water at 20.0°. Combining this value with the unitary free energy of oxygenation of -7.55 kcal/mol of heme (Imai, K. (1973), *Biochemistry 12*, 798) yields an overall entropy change of -22 eu/heme. Experiments carried out in the presence of phosphate or Tris buffers yielded results indicating the existence of pronounced heat effects, in addition to those associated with the release and buffering of oxygen-linked Bohr protons and the binding of oxygen. These uncorrected heat terms are probably attributable to the endothermic release upon oxygenation of exothermically bound buffer ions. Measurements were also made of the fraction of total heat produced as a function of the fraction of total oxygenation. The results were compared with simulated curves in order to place constraints on the heats of binding to the individual sites of the hemoglobin molecule. It was found that a model in which the heats are evenly distributed could not be distinguished from other distributions representing certain limits of variation in individual site heats but that a number of more widely varying models could be eliminated.

Reaction of tetrameric human hemoglobin with oxygen leads to a sequence of events in which approximately 25–30 kcal/mol of heme of free energy is expended upon binding cooperatively to the four heme sites (Roughton and Lyster, 1965; Imai, 1973). Associated alterations in tertiary and quaternary structure lead to a decrease in free energy of interaction between pairs of $\alpha\beta$ dimers amounting to some 8 kcal/mol of heme (Noble, 1969; Thomas and Edelstein, 1972), while release of oxygen-linked Bohr protons occurs with an increase in free energy of about 6 kcal/mol of tetramer

† From the Department of Biochemistry, University of Virginia Charlottesville, Virginia 22901. *Received January 31, 1974.* Support was provided by Grant GM-14493 from the National Institutes of Health.

‡ This work constitutes part of the dissertation presented by D. H. A. in partial fulfillment of the requirements for the Ph.D. degree.

(Antonini *et al.*, 1965). In order to obtain a more complete thermodynamic understanding of these processes it is of interest to separate the free energies into heat and entropy contributions. Comparison between the resulting thermodynamic parameters of various hemoglobins having known structural differences (*i.e.*, mutants and chemically modified hemoglobins) may then lead to a much more detailed understanding of the molecular events responsible for the observed cooperative effects.

The heat of oxygen binding to a number of hemoglobins under various conditions has been studied previously by a combination of direct and indirect methods (Antonini *et al.*, 1965; Benesch *et al.*, 1969; Hill and Wolvekamp, 1936; Roughton, 1935). The most common method has been the indirect van't Hoff determination using spectral measurements. Direct calorimetric determinations have been made by Roughton on the heat of oxygen binding to ox hemoglobin using a gas–liquid calorimeter (Roughton, 1935). The values were compared with van't Hoff determinations under the same conditions, and good agreement was obtained. In addition, he found the heat of oxygen binding to be an approximately linear function of the extent of oxygenation. In contrast, a careful study on sheep hemoglobin (Roughton *et al.*, 1955) showed significant differences in the successive van't Hoff enthalpies for oxygen binding.

No calorimetric studies have been reported to date on oxygen binding to human hemoglobin. Recent studies with horse oxyhemoglobin indicate that determinations carried out in buffer solutions may be complicated by the binding of buffer ions (Hedlund *et al.*, 1972). Corrections for heats of ion binding are not simple to make and have usually been ignored. In this paper we describe calorimetric measurements on the heat of oxygenation of human hemoglobin under simplifying conditions in which no external buffer is present, in order to estimate the intrinsic heat of binding. In addition we report results of determinations in the presence of two buffer systems, which illustrate the existence of pronounced ion binding effects.

A number of observations, such as insensitivity of oxygenation curve shape to temperature, have led to the view that the differences in free energies of binding to the successive sites is predominantly, or entirely, a result of entropy effects, the heats of binding being constant (Antonini *et al.*, 1965; Wyman, 1948). In order to place more accurate constraints on the heats of binding to the individual sites we have measured the heat of binding as a function of the degree of saturation and have compared these curves with possible models for enthalpy distribution with respect to the different sites.

Materials and Methods

Preparation of Hemoglobin. Red cell hemolysates were prepared from freshly drawn citrated blood of healthy human donors. The cells were washed four times in cold isotonic saline by centrifuging at 1000*g* for 10 min at 4° and removing the supernatant after each wash. To the packed cells an equal volume of cold distilled water and 0.5 × original volume of reagent grade toluene was added. The mixture was shaken vigorously at room temperature for 3–5 min. The hemolysates were centrifuged twice at 10,000*g* for 30 min at 4°, with the middle layer of hemoglobin solution carefully transferred to clean tubes. The hemoglobin solution was then dialyzed against 0.05 M Tris (pH 8.0) + 0.1 M NaCl for approximately 12 hr at 6°, then for 12 hr against the same buffer without NaCl.

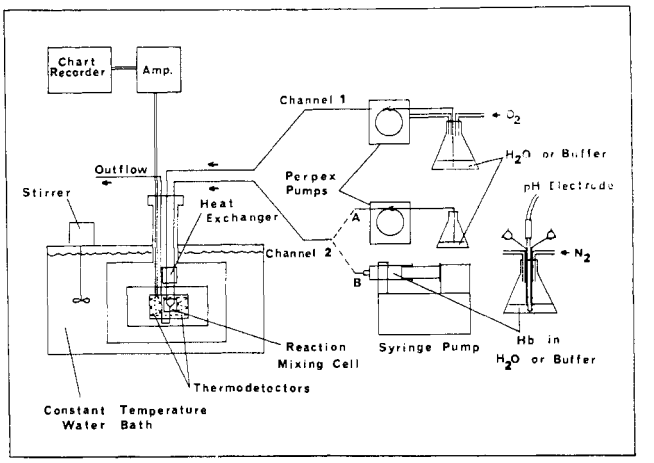


FIGURE 1: Diagram of the apparatus for producing solutions of deoxygenated hemoglobin, measuring their pH, and delivering them to the flow calorimeter for reaction with oxygenated H_2O or buffer. Pump connections at A or B were used interchangeably for base line and test, respectively.

Approximately 50 ml of the dialyzed hemolysate was added to a DEAE A-50 Sephadex ion-exchange column (5 × 60 cm), previously equilibrated at 6° with 0.05 M Tris (pH 8.3) at a flow rate of approximately 150 ml/hr. The first band was removed by elution with 0.05 M Tris (pH 8.0) and the major portion of the main component, hemoglobin A, was collected by elution with the same buffer at pH 7.6. This fraction was then concentrated to approximately 50 mg/ml using an Amicon column eluate concentrator and dialyzed for 24 hr at 6° against two changes of deionized and distilled water.

Just prior to use, hemoglobin solutions were deionized by passage at 6° at approximately 10 ml/min, through an Amberlite MB-1 ion-exchange column (3 × 40 cm), previously washed with several volumes of deionized distilled water. The leading portion of the hemoglobin was collected and stored at 4°. The percentage of methemoglobin, determined by the method of Benesch *et al.* (1965), was less than 3%.

Calorimetric Measurements. A specially modified LKB flow calorimeter was used in these studies. The calorimeter head No. 10711 with cells, detectors, and heat sink was obtained from LKB, housed in a specially designed watertight container (Zimmer, 1971) and submerged within a Tronac 1005 temperature-controlled water bath with a Tronac 1040 proportional temperature controller. All calorimetric measurements were made at 20.0°. Electrical calibration was made using an LKB 10700 control unit. A Keithley 150B microvoltammeter was used to amplify and a Sargent-Welch SRG strip-chart recorder was used to display the resulting signal. In addition to electrical calibration, the signal was calibrated chemically by protonation of Tris (Ultra Pure from Schwarz/Mann) using the literature value of -11.32 ± 0.06 kcal/mol for the heat of protonation (Wadsö, 1968). Figure 1 describes the apparatus used to generate solutions of deoxygenated hemoglobin and oxygenated water or buffer and deliver them to the reaction mixing cell inside the calorimeter. To ensure a gas tight system, the tubing and flow connections were made of glass and Tygon covered Teflon in the short distances where glass connections were impractical. Two LKB 10200 Perpex pumps at a flow rate of 16.1 ml/hr were used with a connection to oxygen for the pump used in channel 1 to ensure that the water or buffer remained under a 100% oxygen atmosphere. A Sage 225-2 syringe pump, equipped with a Hamilton gastight syringe, set at the same flow rate, was used interchange-

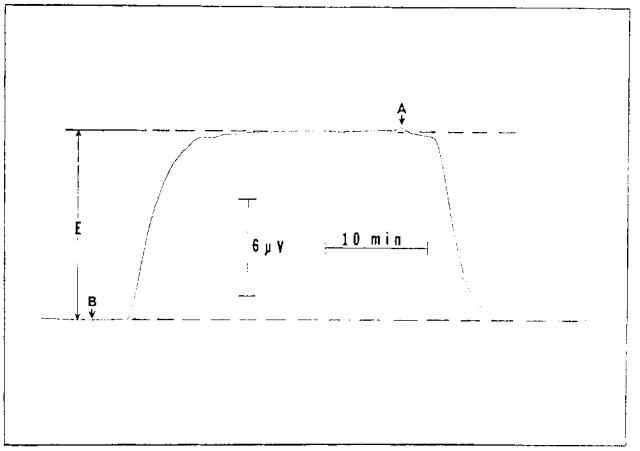


FIGURE 2: Chart recording of the signal produced by the heat of oxygenation of human hemoglobin at 20.0° in H_2O at a rate of 1.8×10^{-9} mol of heme/sec. Points marked A and B are explained in text.

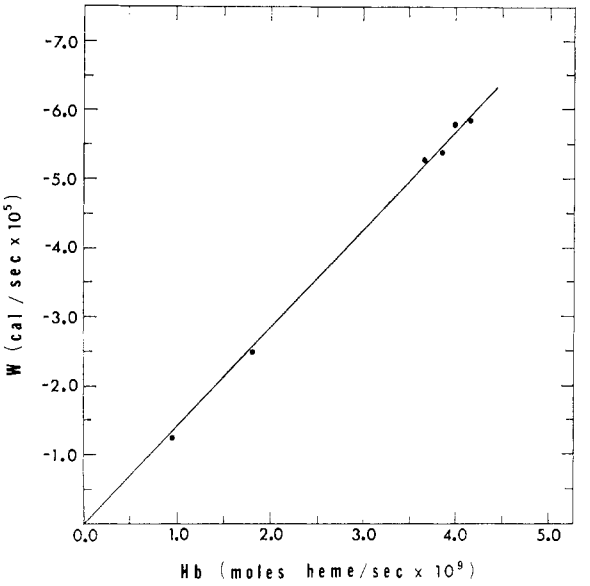


FIGURE 3: Concentration dependence of the heat of oxygenation of human hemoglobin at 20.0° in H_2O. The line is the least-squares fit to the data through the origin with a slope of -14.1 ± 0.4 kcal/mol of heme.

ably with the Perpex pump in channel 2 when a deoxygenated solution of hemoglobin was to be reacted in the calorimeter.

Hemoglobin solutions at concentrations ranging from 3 to 15 mg per ml were prepared by equilibrating them under nitrogen in a 500 ml Erlenmeyer flask equipped with a pH electrode, a syringe connection for withdrawing the hemoglobin, and a connection for the addition of titrating acid or base to adjust the pH. This reservoir and the reservoir in channel 1 were placed at room temperature on a slowly oscillating platform to increase the rate of equilibration. The nitrogen used was purified of oxygen by passing it through a chain of vanadous chloride (Meites and Meites, 1948). pH measurements were made at 22.5° on a Radiometer Model 26 expanded scale pH meter.

The fraction of total oxygenation was measured on a Cary 14 spectrophotometer equipped with a 1.0-mm flow cell with gas tight connections and thermostated at 20.0°. The absorbances at 540, 560, and 576 nm were taken after a sample of hemoglobin from a Hamilton syringe was introduced. The fraction of total oxygenation and the per cent methemoglobin (which remained below 3%) were computed using the equations of Benesch *et al.* (1965). The syringe was then placed in the Sage pump and connected to the calorimeter so that a remaining portion could be used for calorimetric measurements. Final determinations of the percentages of oxygenation and methemoglobin in the syringe and at the outflow of the calorimeter showed that deoxyhemoglobin could be completely oxygenated in the calorimeter using this system, and that less than 2% of deoxyhemoglobin was converted into methemoglobin or oxyhemoglobin, *e.g.*, by oxygen leaks.

Results and Discussion

Calibration. Electrical calibration curves at 20.0° were obtained for the range of 0–10 mcal/sec with a calibration constant of $3.90 \pm 0.01 \times 10^{-6}$ cal/sec μV. This constant was used for the calorimetric measurements. A constant of $3.91 \pm 0.01 \times 10^{-6}$ cal/sec μV was obtained from the chemical calibration for the same temperature.

Measuring the Heat of Oxygenation. Figure 2 shows the signal obtained from the reaction of deoxygenated hemoglobin at a rate of 1.8×10^{-9} mol of heme/sec. The base line is obtained by connecting channel 2 (as shown in Figure 1) to the Perpex pump at A, which delivers water or buffer at a flow rate as close as possible to that of channel 1. At the point marked B (Figure 2), the syringe containing the hemoglobin solution is connected to the calorimeter. The resulting heat of that reaction is measured as the final amplitude marked E. At point A the calorimeter is again connected to the Perpex pump and the recorder returns to base line. The heat of dilution of hemoglobin in deoxygenated water was found to be essentially zero under the conditions of these experiments, and no correction was necessary for this effect.

Figure 3 shows the concentration dependence of the heat of oxygenation of human hemoglobin in deionized distilled water at 20.0°. Calculations showed that the amount of oxygen in solution in channel 1 available to react with the deoxyhemoglobin in channel 2 is adequate to completely saturate hemoglobin solutions in this concentration range, as indicated by a straight-line fit of the data. The straight line shown is the least-squares fit through the origin which gives a value computed from the slope of -14.1 ± 0.4 kcal/mol of heme. Two of the four points at the upper end of the curve, on alternate sides of the line, are heats obtained in separate experiments using a different preparation of hemoglobin. Within experimental error, purified isoionic hemoglobin A has the same heat of oxygenation per mole of heme as that of dialyzed deionized hemoglobin.

Interpreting the Heat of Oxygenation under Various Buffering Conditions. In Table I are shown values for the measured heats of oxygenation, $\Delta H'$, of human hemoglobin A determined at different pH's and under different buffering conditions. In order to interpret these data it is necessary to recognize that the observed heat $\Delta H'$ is a sum of heats which may be described by eq 1, where $\overline{\Delta x}$ = moles of Bohr protons released upon oxygenation; ΔH_i = heat of proton release of the oxygen-linked Bohr protons; ϕ = fraction of Bohr proton buffering contributed by foreign buffer; ΔH_b = heat of ionization of foreign buffer; ΔH_s = heat of ionization of the buffering groups on the protein; ΔH_{bb} = heat of binding of buffer

$$\Delta H' = \overline{\Delta x}\Delta H_i - \overline{\Delta x}[\phi\Delta H_b + (1 - \phi)\Delta H_s] + \psi\Delta H_{bb} + \Delta H_o \quad (1)$$

TABLE I: Heat of Oxygenation of Human Hemoglobin.

Run	Buffer Conditions			ΔH_b[c] (kcal/mol)	Obsd Heat[d] (kcal/mol of Heme), $\Delta H'$	App Buffer Binding Heat[e] (kcal/mol of Heme), $\psi\Delta H_{bb}$
		pH	$\overline{\Delta x}$[a]	ϕ[b]			
1	H_2O	7.5	0.55	0	0	−14.2	0
2	H_2O + NaOH	8.5	0.20	0	0	−14.2	0
3	H_2O + NaOH	9.5	0	0	0	−13.9	0
4	0.1 M Tris	7.5	0.55	1	11.3	−14.0	3.0
5	0.1 M Tris	9.5	0	1	11.3	−13.8	0.1
6	0.1 M phosphate	7.5	0.55	1	1.1	−7.0	4.6
7	0.1 M phosphate	9.5	0	1	1.1	−13.2	0.7

[a] Number of equivalents per heme of Bohr protons released upon oxygenation. [b] Fraction of total buffering of Bohr protons contributed by foreign buffer. [c] Heat of ionization of foreign buffer. [d] Measured heat at 20.0° at a protein concentration of 0.9 mM heme (14.5 mg/ml). Values are accurate to ±0.4 kcal/mol of heme. [e] The difference between heats of buffer ion binding in the oxy and deoxy forms of hemoglobin calculated from eq 3. Values are accurate to ±0.4 kcal/mol of heme.

ions (or other solute species) to the protein; ψ = difference between the number of moles of buffer ion (or other species) bound in the oxy and deoxy forms of the protein; ΔH_o = intrinsic heat of binding. This term represents the observed heat of oxygen binding corrected for ionization and ion binding effects. It includes the following heats: (1) heat of oxygen binding to the heme sites; (2) heats of conformational and quaternary structure change associated with binding (*e.g.*, the breaking of salt bridges (Perutz, 1970)); (3) any difference between heat content of the oxy and deoxy forms of the protein. The simplest case, corresponding to runs 1–3 (Table I), is that in which no external buffer ions are present (ϕ and ψ are zero) so that the measured heat reduces to

$$\Delta H' = \Delta H_o + \overline{\Delta x}(\Delta H_i - \Delta H_s) \quad (2)$$

At pH 9.5 (run 3) the Bohr effect is essentially absent ($\overline{\Delta x} = 0$) so that the measured heat $\Delta H'$ is equal to ΔH_o, the intrinsic heat of binding. The values of $\overline{\Delta x}$ listed in Table I are taken from the titration results of Antonini *et al.* (1965). They found that the Bohr effect can be conveniently described in terms of two kinds of ionizable groups having heats of proton release of +9.0 and −1.5 kcal per mol. In the pH region of 7.0–8.5 the net heat of Bohr proton release (5.8 kcal/mol) is very close to the +6.5 kcal/mol attributed earlier to deprotonation of a single histidine imidazole (Antonini *et al.*, 1965). From the titration curves it is also evident that the buffering groups of the hemoglobin molecule in this pH region are the histidines.[1] Consequently ΔH_i and ΔH_s will be very nearly equal in each of the experiments at pH 7.5 and 8.5. The measured $\Delta H'$ may be regarded as essentially identical with the intrinsic heat of binding under these conditions (runs 1 and 2, Table I). The errors of this approximation are less than the errors of the $\Delta H'$ measurements, which are accurate to ±0.4 kcal/mol. Within these limits the constancy of $\Delta H'$ in runs 1, 2, and 3 provides support to the proposal of Wyman that the intrinsic heat of binding is essentially independent of pH (Wyman, 1948).

In the presence of foreign buffer the situation is more complex. This is shown by the results obtained in 0.1 M Tris-HCl and 0.1 M potassium phosphate (runs 4–7, Table I). In these experiments the buffering capacity of the external buffer is much greater than that of the protein so that ϕ is unity and

$$\Delta H' = \Delta H_o + \overline{\Delta x}(\Delta H_i - \Delta H_b) + \psi\Delta H_{bb} \quad (3)$$

Neither ψ nor ΔH_{bb} is known. However, the product $\psi\Delta H_{bb}$ may be calculated in each case from the measured heats using the intrinsic enthalpy ΔH_o determined in runs 1–3, the known ionization enthalpy ΔH_b for the buffer used, and the values of $\overline{\Delta x}$ and ΔH_i from the titration results of Antonini *et al.* (1965). The resulting values for the "buffer binding heats" are shown in Table I.

Although an exact accounting for the sources of this apparent buffer binding heat would require more extensive studies, the endothermic values may be interpreted as probably arising from the release of exothermically bound ion species upon oxygenation. It is seen that the effect is greatest in phosphate buffer at pH 7.5, as would be expected from the strong linkage between phosphate binding and oxygenation. This interpretation is also consistent with the observations of Hedlund *et al.* (1972) who found greatly decreased negative heats of ATP binding to horse oxyhemoglobin in the presence of chloride or phosphate ions. Although very few instances of effect have been studied to date, one would suspect it to be widely exhibited by proteins in which cooperative transitions in conformation are linked to binding of small molecules. This possibility necessitates severe caution in the use of buffer pairs to correct for ionization effects in calorimetric experiments. Use of this common procedure, for example, with the data of runs 4 and 6 would yield the simultaneous equations (*i.e.*, ignoring ΔH_{bb} in eq 3)

$$-14.0 = \Delta H_o + \overline{\Delta x}(5.8 - 11.3)$$
$$-7.0 = \Delta H_o + \overline{\Delta x}(5.8 - 1.1)$$

from which we would calculate $\overline{\Delta x}$ and ΔH_o to be 0.69 and −10.2, respectively. The values for ΔH_b, the ionization enthalpies for Tris and phosphate buffers, were taken from the literature (Langerman and Sturtevant, 1971).

For comparison with results of this study, we have listed values for the van't Hoff heats of oxygenation of human hemoglobin preparations (Table II) which were taken from the literature and corrected for the heat of solution of oxygen by using a value of −3.0 kcal/mol of O_2 (Antonini and Brunori, 1971). This correction is not necessary for the calorimetric

[1] Self-buffering by the protein is essentially complete. When the experiment is carried out in deionized water with a deoxyhemoglobin solution initially at pH 7.5, the pH was found to decrease by 0.23 pH unit upon oxygenation. At the protein concentrations used in these experiments, the fraction of Bohr protons which remain unbuffered by the protein is approximately 10^{-4}.

TABLE II: Literature Values for van't Hoff Heat of Oxygenation of Human Hemoglobin.

Preparation	Buffer	pH	$\Delta H'$ [a] (kcal/mol of O_2)	Reference
Hemolysate	0.1 M phosphate	7.3	−7.0	Hill and Wolvekamp (1936)
Hb A	0.05 M Bis-Tris	7.3	−10.7	Benesch *et al.* (1969)
Hb A	2% borate	9.5	−11.5	Antonini *et al.* (1965)
α, β chains	0.1 M phosphate	7.0	−10.5	DeRenzo *et al.* (1967)

[a] Corrected for heat of solution of oxygen (−3.0 kcal/mol of O_2).

measurements. It is seen that the van't Hoff heats are all lower than the intrinsic heat ΔH_o obtained calorimetrically in the present investigation. This is to be expected in view of the results discussed above.

The intrinsic enthalpy determined in this study can be combined with the corresponding free energy estimated from Imai's data (Imai, 1973) to calculate the overall entropy of complete oxygenation. His binding constants in 0.01 M Tris, with no additional salt, correspond most closely to the conditions under which ΔH_o has been obtained in this study. Using the Gibbs-Helmholtz equation and correcting the 25° data to 20° yield a unitary free energy for total oxygenation of −7.55 kcal/mol of heme. From this value and the enthalpy determined from calorimetric data, the entropy change for total oxygenation of the hemoglobin tetramer is −22 eu/heme.

In a recent calorimetric study of the Bohr effect for the reaction of human hemoglobin with carbon monoxide (Rudolph and Gill, 1973), a constant value for the intrinsic heat of reaction, after correcting for ionization processes in the protein, was found to be −23.2 ± 1.5 kcal/mol of CO over the range pH 6–9 at 25°. This result is in excellent agreement with our studies, after correcting for the heat of solution of carbon monoxide (−2.9 kcal/mol of CO) and the heat for O_2 replacement by CO (−5.0 kcal/mol of CO; Antonini and Brunori, 1971).

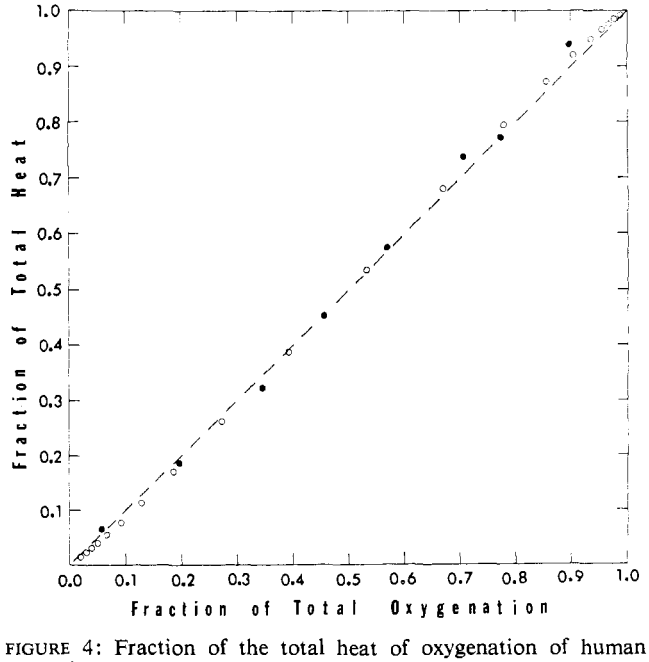


FIGURE 4: Fraction of the total heat of oxygenation of human hemoglobin, at 20.0° in H_2O, as a function of the fraction of total oxygenation. The protein concentration is 0.93 mM heme (15 mg/ml). The darkened points are experimental data. The open points are simulated heats where $\Delta H_1 = -9$ kcal/mol, $\Delta H_2 = -19$ kcal/mol, $\Delta H_3 = -21$ kcal/mol, and $\Delta H_4 = -7$ kcal/mol, obtained by a computer analysis of the data using the oxygen binding constants of Imai (1973). See text for details.

Estimating the Heat of Oxygen Binding to Successive Sites. Values for the amount of heat obtained from solutions of isoionic human hemoglobin in deionized distilled water at varying states of oxygenation are shown by the darkened points in Figure 4. The fraction of the total heat of oxygenation f_o for each point was obtained using

$$f_o = 1 - (Q/Q_o) \quad (4)$$

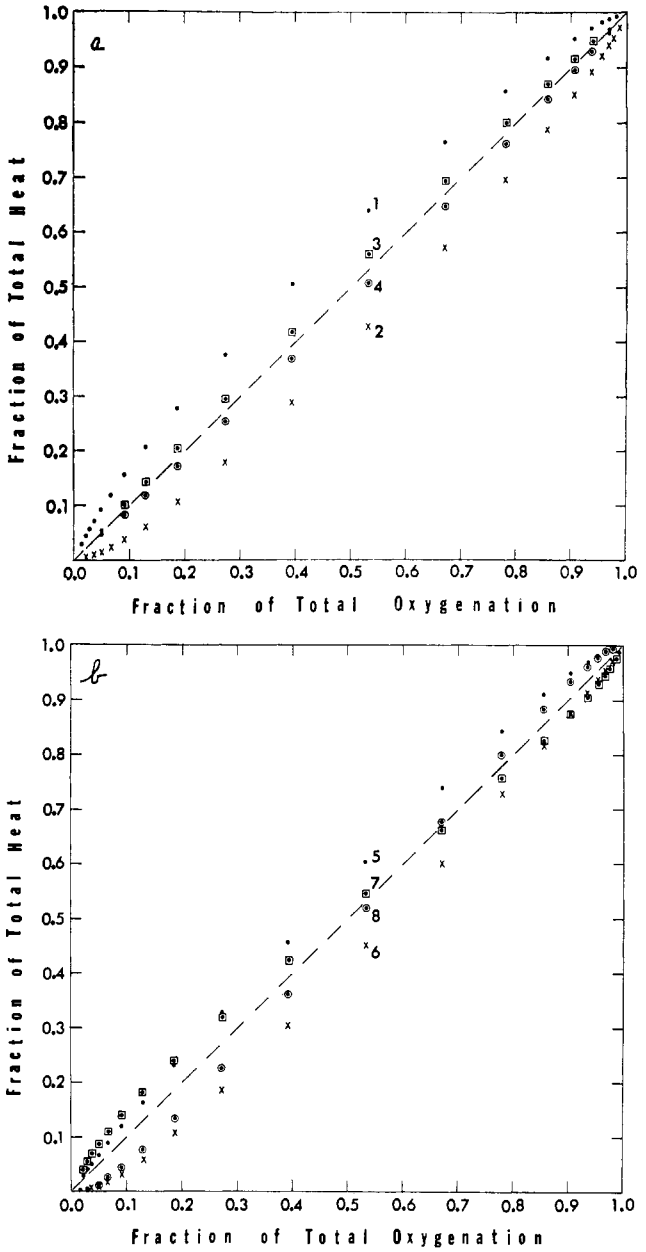
where Q is the heat produced by completely oxygenating a hemoglobin solution from a particular state of oxygenation and Q_o is the total heat obtained by completely oxygenating a solution from the deoxy state. The open points in Figure 4 were obtained by fitting the data by a computer analysis using equilibrium constants for the binding of oxygen to human hemoglobin A in 0.01 M Tris (pH 7.3) obtained by Imai (1973). This analysis produced values of $\Delta H_1 = -9 \pm 5$ kcal/mol, $\Delta H_2 = -19 \pm 12$ kcal/mol, $\Delta H_3 = -21 \pm 12$ kcal/mol, and $\Delta H_4 = -7 \pm 6$ kcal/mol heats of binding to the successive sites on the hemoglobin molecule. From these values and the Adair constants one can calculate f_o as a function of the degree of oxygenation. These values are represented by the open points of Figure 4. The broken line of Figure 4 represents the fraction of total heat that would be obtained if the heats of binding to the successive sites were identical.

In order to explore further the sensitivity of the calorimetric titration to different models for heat distribution, a number of cases were simulated, as listed in Table III. Figure 5a–c

TABLE III: Distribution of Total Enthalpy for Successive Oxygen-Binding Steps in Heat Curve Simulations (Figures 5a–c).

Simulation	$\Delta H_1/\Delta H_T$ [a]	$\Delta H_2/\Delta H_T$	$\Delta H_3/\Delta H_T$	$\Delta H_4/\Delta H_T$
1	0.50	0.25	0.20	0.05
2	0.05	0.20	0.25	0.50
3	0.25	0.50	0	0.25
4	0.25	0	0.50	0.25
5	0.333	0.333	0.333	0
6	0	0.333	0.333	0.333
7	0.50	0	0	0.50
8	0	0.50	0.50	0
9	0.50	0.167	0.167	0.167
10	0.167	0.167	0.167	0.50
11	0.167	0.50	0.167	0.167
12	0.167	0.167	0.50	0.167

[a] ΔH_T = total heat for complete oxygenation.



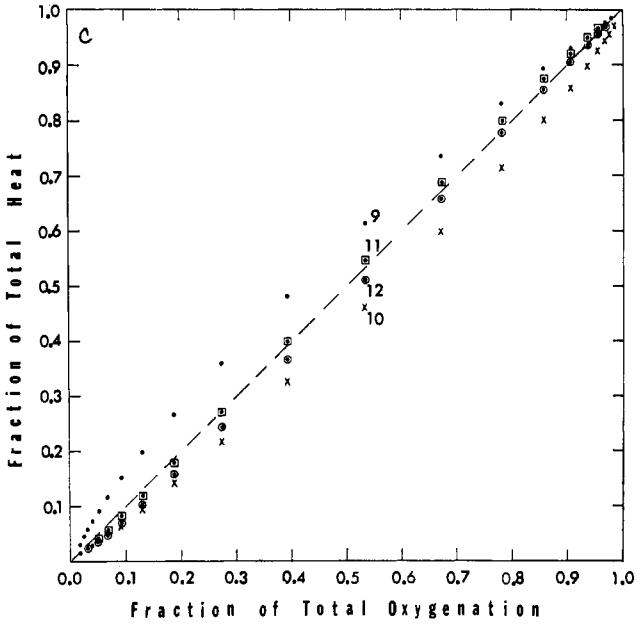


FIGURE 5: Simulated oyxgen-binding heat curves using distributions of the fractional heats of binding for the individual sites on the hemoglobin molecule as listed in Table III.

shows simulated values for the fraction of total heat that would be obtained for the various distributions of the heats of binding. It can be seen that the experimental data clearly impose constraints on the possible distributions of binding heat to the individual sites. Although there is scatter in the data, particularly at higher fractions of total heat, where measurement according to eq 4 is most subject to error, all the distributions in Table III can be eliminated except simulations 3, 4, 11, and 12. It is much more difficult to put constraints on the heats of binding to the second and third sites than those of the first and fourth due to the low concentrations of the doubly and triply liganded species, as determined by the binding constants of Imai (1973). The results would be qualitatively similar using binding constants for a statistical distribution of species in the absence of cooperativity. From these results it is clear that the heat of oxygenation of the hemoglobin tetramer as a function of per cent oxygenation can be described as a summation of approximately equal heat contributions. However it is also possible that differences approximating a factor of two may exist in the individual heats, as illustrated by cases 3, 4, 11, and 12. The present results do not distinguish between possibilities within these limits. They do, however, rule out more extreme variations, represented by the other cases of Table III.

Of particular interest for future measurements would be a calorimetric determination of the intrinsic heat of oxygenation of isolated α and β chains. Comparison with the heat found in the intact hemoglobin tetramer would provide further evidence on the question of whether the heats of binding to the individual subunits in hemoglobin are equivalent, and not affected by the quaternary state of the molecule.

Acknowledgment

We thank Dr. Austen F. Riggs for the hemoglobin preparation procedure used in this work, and Dr. Michael L. Johnson for writing the computer program for analyzing the calorimetric data.

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Effect of Magnesium Adenosine 5′-Triphosphate on the Accessibility of the Iron of Clostridial Azoferredoxin, a Component of Nitrogenase†

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ABSTRACT: Azoferredoxin has been shown to have a minimum of three different interconvertible states: an oxidized state (one in which its activity is not destroyed), a reduced state, and a reduced state in the presence of magnesium ATP. Each of these states could function in the reductions catalyzed by nitrogenase. The rapid removal of iron from azoferredoxin by α,α'-dipyridyl, only when azoferredoxin is complexed with magnesium ATP, is directly correlated with a loss in the electron paramagnetic resonance spectrum of the reduced azoferredoxin–magnesium ATP state. In the absence of α,α'-dipyridyl, the effect of magnesium ATP is reversible. The magnesium ATP effect is highly specific since no other magnesium nucleotide examined catalyzed the rapid transfer of iron from azoferredoxin to α,α'-dipyridyl. In addition, the iron of molybdoferredoxin, the other nitrogenase component, is not made accessible to α,α'-dipyridyl upon addition of magnesium ATP. Although ADP has been shown to compete with ATP in the nitrogenase system and to inhibit its activity, it does not mimic ATP in allowing the iron of azoferredoxin to be removed by added iron chelators. In fact, ADP inhibits the ability of ATP to make the iron of azoferredoxin accessible to chelators. Significantly, reduced molybdoferredoxin which itself reacts very slowly with α,α'-dipyridyl also hinders the removal of iron from the magnesium ATP complex of azoferredoxin. Oxidation of azoferredoxin in such a way that most of it is not inactivated disrupts its α,α'-dipyridyl-resistant structure. However, if dithionite is immediately added to the protein after oxidation, that protein not inactivated returned to its α,α'-dipyridyl-resistant structure. The amount of restoration correlated with the amount of activity recovered after the same treatment.

ATP hydrolysis is required in the catalytic transfer of electrons by nitrogenase from a reductant to dinitrogen (McNary and Burris, 1962; Mortenson, 1964; Hardy and D'Eustachio, 1964). Neither component of the enzyme, azoferredoxin (iron–protein) or molybdoferredoxin (molybdenum–iron protein), is enzymatically active in the absence of the other (Mortenson *et al.*, 1967). It is not understood how ATP functions in the reduction of substrates by nitrogenase, although several hypotheses have been advanced. Among these postulates are: (1) electron activation by ATP (Mortenson, 1964; Hardy *et al.*, 1965), (2) ATP mediated electron transfer (Mortenson, 1964; Hardy and Burns, 1968), (3) ATP supported substrate binding (Kelly and Lang, 1970), and (4) ATP induced changes in the conformation of one or both components of nitrogenase (Bulen *et al.*, 1965b; Silverstein and Bulen, 1970; Yates, 1972; Zumft *et al.*, 1973).

Zumft *et al.* (1973) proposed that ATP induces a conformational change in azoferredoxin because the changes that it caused in the electron paramagnetic resonance (epr) spectrum of azoferredoxin were similar to those spectral changes obtained with azoferredoxin in the presence of 5 M urea. These observations suggested that a urea- or ATP-induced conformational change would probably involve a reorientation of the iron–sulfur center(s) within azoferredoxin. Walker and Mortenson (1973) recently reported that the iron in reduced azoferredoxin is accessible to an iron chelator in the presence of MgATP[1] but not in its absence. The latter provided strong evidence that MgATP alters the conformation of azoferredoxin.

This study elaborates the effect of MgATP on azoferredoxin in the presence of the ferrous iron chelator, α,α'-dipyridyl.

Materials and Methods

Azoferredoxin (AzoFd) and molybdoferredoxin (MoFd) were prepared from *Clostridium pasteurianum* W5 by the method of Zumft and Mortenson (1973). Final preparations of AzoFd and MoFd were in 0.05 M Tris-HCl (pH 7.4) and were 0.35 and 0.25 M, respectively, in NaCl and 1 mM with

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[1] Abbreviations used are: MgATP, for the negatively charged complex ($Mg \cdot ATP^{2-}$) which exists under the conditions employed in these experiments; AzoFd, azoferredoxin; MoFd, molybdoferredoxin.