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# Thin-layer microcalorimetric studies of oxygen and carbon monoxide binding to hemoglobin and myoglobin

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**Abstract**

A thin-layer gas-solution microcalorimeter has been developed to study the binding reactions of gaseous ligands with ligand binding macromolecules. We have measured the enthalpy of binding oxygen and carbon monoxide to horse myoglobin, human hemoglobin $A_0$ and sperm whale myoglobin in phosphate buffer at pH 7.6, with the enzyme reducing system of Hayashi [1]. Reactions of human hemoglobin were also done under various buffer conditions in order to elucidate the Bohr effect. These binding reactions were found not to exhibit a detectable enthalpy change over the temperature range of 10°C to 25°C. The enzyme reducing system was shown to react with oxygen in a manner that releases a substantial amount of heat. This problem was corrected by using a minimum amount and by placing the buffer and enzyme system in the reference cell effectively canceling the oxygen enzyme reaction heat as well as the heat of gas dissolution. It was also demonstrated that glucose-6-phosphate, one of the reducing system components, in 50 m $M$ concentrations can influence the heat of binding oxygen and carbon monoxide to hemoglobin. This effect was shown to be absent in the myoglobins and also with hemoglobin at glucose-6-phosphate concentrations less than 5 m $M$.

*Keywords:* Hemoglobin; Myoglobin; Thin-layer microcalorimeter; Oxygen binding; Carbon monoxide binding

## 1. Introduction

The thermodynamic characterizations of ligand binding reactions of oxygen with hemoglobin have normally been pursued by analyzing ligand binding curves within the Adair formalism. The Adair coefficients provide the free energy changes associated with successive steps in oxygenation; the separation of the free energy changes into enthalpic and entropic contributions is more problematic. Application of the van 't Hoff relationship to the temperature dependence of the free energy changes results in an enthalpic–entropic separation. However, caution must be exercised, for this analysis assumes that the change in heat capacity of reaction associated with ligand binding is zero, in other words the enthalpy of reaction is independent of temperature. A second assumption for optical methods, is that there is a

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Abbreviations used: M = subunit containing a heme, Mb = myoglobin, Hb = hemoglobin, $O_2$ = oxygen, CO = carbon monoxide, ENZ = enzyme reducing system.

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Table 1

Table of previously reported enthalpies (kcal/mol heme)

| Species | Reaction | | | Ref. |
|---|---|---|---|---|
| | $M+CO \rightarrow MCO$ | $M+O_2 \rightarrow MO_2$ | $MO_2+CO \rightarrow MCO+O_2$ | |
| Sperm whale | −21.2 | −17.9 | – | Keyes et al., 1971 [14] |
| myoglobin | −17.7 | – | – | Rudolph et al., 1972 [15] |
| Human | – | −13.2 | – | Roughton, 1935 [16] |
| hemoglobin $A_0$ | – | −13.4 | – | Splittgerber, 1968 [17] |
| | – | −15.7 | – | Bucci et al., 1991 [18] |
| | −12.4 | −7.7 | – | Parody-Moreale et al., 1987 [19] |
| | −15.0 | – | – | Gill et al,. 1980 [20] |
| | – | −8.6 | – | Mills and Ackers, 1979 [13] |
| | – | −12.3 | – | Imai, K. 1979 [21] |
| | −17.2 | −13.2 | −4.0 | Gaud et al., 1974 [22] |
| | – | −7.0 | – | Atha and Ackers, 1974 [23] |
| | – | −11.7 | – | Imai and Tyuma, 1973 [24] |
| Myoglobin | −16.2 | – | – | Rudolph et al., 1972 [15] |
| | −22.3 | −17.5 | – | Theorell, 1934 [25] |
| Human myoglobin | – | −13.0 | – | Rossi-Fanelli and Antonini, 1958 [26] |

linear optical response upon ligand binding which is temperature independent.

An alternative approach to the van 't Hoff method for obtaining enthalpies of ligand binding is to directly measure the enthalpy change upon ligand binding by calorimetry. To this end we have developed a thin layer gas-solution microcalorimeter which utilizes millimolar concentrations of heme in microliter quantities while exhibiting microjoule sensitivity. In this paper we report the development of this calorimeter and an initial study of the temperature dependence of the overall binding enthalpies of oxygen and carbon monoxide with sperm whale and horse myoglobin, and human hemoglobin. Table 1 contains previously determined van 't Hoff and calorimetric values for the binding of carbon monoxide and oxygen to hemoglobin and myoglobin.

## 2. Materials and methods

### 2.1 General preparations

Horse and whale Met myoglobin samples were purchased from Sigma. The solutions were filtered (0.45 $\mu$m) and dialysed at 6°C for 48 hours in four separate six liter solutions of 0.1 *M* potassium phosphate and 1 m*M* EDTA at pH 7.6. Human Hemoglobin $A_0$ samples, stripped of organic phosphates, were prepared according to the method reported by Williams and Tsay [2]. The resulting hemoglobin, less than three percent met, was then dialyzed at 5°C for 36 hours in three separate six-liter solutions of 0.2 *M* potassium phosphate and 1 m*M* EDTA at pH 7.6. The phosphate buffer was chosen for its small protonation heat of approximately −0.8 kcal/mol [3]. Purified protein samples were stored as pellets in liquid nitrogen until ready for use. Enzyme reducing system components we. purchased from Sigma and placed in the appropriate buffers before addition to the heme solutions. The samples were checked for alteration in pH before beginning the experiment.

### 2.2 Concentration determination

Hemoglobin and myoglobin concentrations were determined using a previously calibrated thin-layer optical cell (0.0101 cm) placed in a Cary 219 spectrophotometer. Hemoglobin extinction coefficients were taken from the values of Benesch et al. [4] modified by van Assendelft and Zijlstra [5]. A computer program based on these values was written to determine the concentra-

tions of oxygenated, deoxygenated and met hemoglobin components. The appropriate myoglobin extinction coefficients were taken from Antonini [6] and Antonini and Brunori [7]. Concentrations of myoglobin were determined in the fully oxygenated reduced forms. The oxy-horse myoglobin millimolar extinction coefficient at 580 nm is 14.4 $mM^{-1}$ $cm^{-1}$ and the oxy-sperm whale myoglobin at 581 nm is 14.6 $mM^{-1}$ $cm^{-1}$. Hemoglobin concentrations were approximately 4 m$M$ heme, while those for myoglobin were 2.5 m$M$ heme. Precise concentrations were determined to three significant figures after the addition of enzyme reducing system just before the beginning of the experiment.

### 2.3 Thin-layer calorimetry

The calorimeter (Fig. 1), an extensive modification of an earlier version, consists of two aluminium blocks shielded by an aluminium case enclosed in a CPVC canister [8]. Two thin-walled flat-bottom NMR tubes, acting as cell holders, extend through the top aluminium block into close fitting copper cups mounted on thermopiles that are hooked in opposition (twinned). The bottom aluminium block contains spring loaded aluminium pistons serving to position the thermopiles with constant force against the copper cups, making it easier to assemble. Nominal 100-ohm resistor wires are wrapped around the outside of the copper cups and computer controlled thermo-electrical compensation is employed. A stainless steel dilution valve at the top allows for the titration of gaseous ligands by way of successive logarithmic dilutions. In this set of experiments it is used simply to supply an excess of reacting ligand. The valve is equipped with an oxygen electrode in order to monitor the activity of oxygen and check the system for leaks. Temperature is controlled by a Tronac regulated water bath with deviations less than $\pm 0.0003$°C.

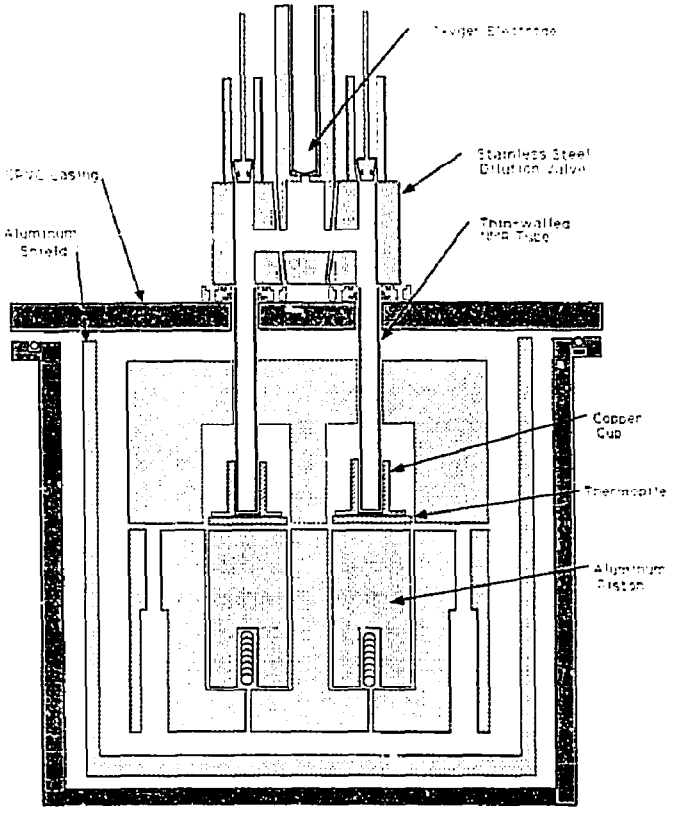


Fig. 1. Calorimeter and gas dilution valve in detail.

The instrument is calibrated electrically by placing a nominal 100 ohm resistor wire wrapped around a thin-walled copper dowel inserted into an NMR tube cell (Fig. 2). This cell, which mimics the sample orientation, is placed into the calorimeter. A series of calibration heats are run, the cell constant was determined to be $2.99 \pm 0.02$ joules per volt second with a 0.43% heat loss.

The sample and reference solutions, measured by Hamilton syringe (typically 50.0 $\mu$l), are suspended on a piece of filter paper that is placed on the inside wall of a flat bottom modified NMR tube approximately 2.5 cm in length and 7 mm in diameter (Fig. 2). These close-fitting cells are then inserted into the calorimeter so that they extend into the region of the copper cups. In the case of carbon monoxide or oxygen binding to deoxy heme the sample and reference are flushed with buffer equilibrated nitrogen until oxygen is removed, this procedure taking up to five hours for the high affinity horse Mb at low temperatures. The bore of the valve is then flushed with buffer equilibrated carbon monoxide or oxygen, the valve is turned and reacting ligand is released into the reaction chambers. In the replacement reaction in which oxygen is displaced by carbon monoxide, the system is flushed with buffer equi-

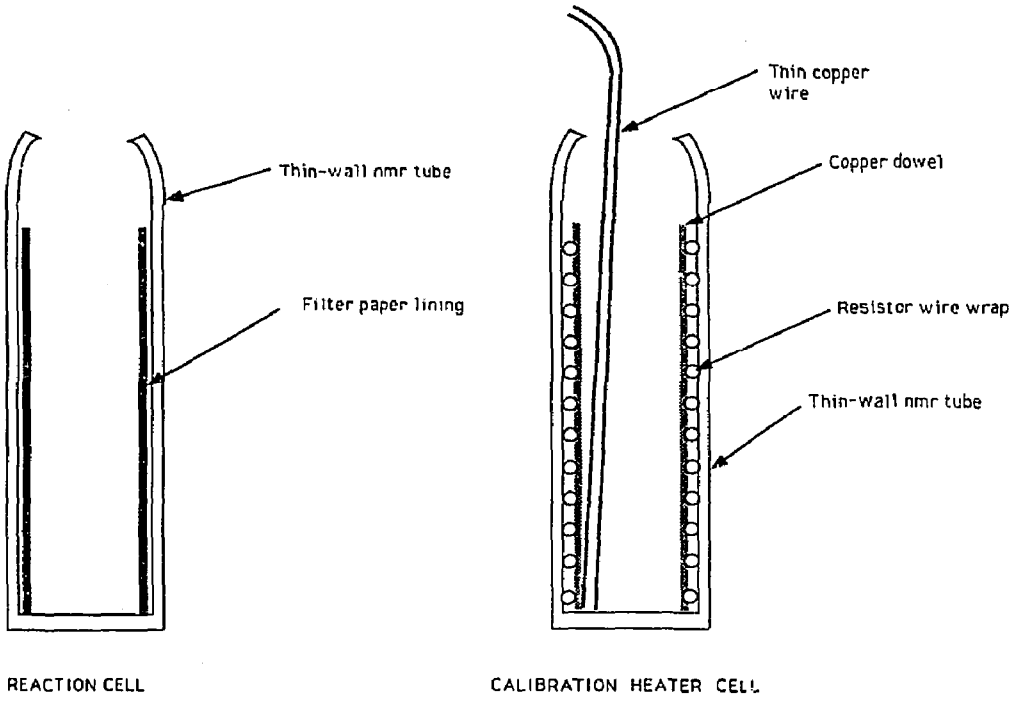


Fig. 2. Modified NMR tube cell and calibration heater. Cells are rounded at the top in order that they may be easily removed from the calorimeter.

librated oxygen and then titrated with carbon monoxide. All of these types of reactions are completed in less than five minutes and 95% electrical compensation is obtained. The heat effect caused by the dissolution of gas is canceled out by the placement of buffer in the reference cell. Reactions conducted with a number of different filter papers at varying concentrations of heme indicated no unusual filter paper interactions.

## 3. Results and discussion

In order to limit the extent of met heme production the enzyme system of Hayashi was used (Fig. 3) under conditions shown in Table 2 [1]. The components for the reducing system include an NADPH generating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase) and an electron-mediating system (ferredoxin and ferredoxin-NADP reductase). Together, along with catalase (used to eliminate peroxides), this ensemble serves to keep hemoglobin and myoglobin in their reduced forms in a continuous fashion with glucose-6-phosphate acting as the fuel. The reducing system is thought to accomplishes this without alteration or damage to the reduced protein. However, two problems were manifested when reacting carbon monoxide and oxygen with myoglobin and later with hemoglobin. The first situation was encountered when reacting sperm whale myoglobin and enzyme system with carbon monoxide while placing only buffer in the reference cell. For the binding of CO,

$$Mb + CO \rightarrow MbCO$$

$$\Delta H = -17.3 \pm 0.05 \text{ kcal/mol heme}$$

When the replacement reaction was carried out,

$$MbO_2 + CO \rightarrow MbCO + O_2$$

$$\Delta H = -1.32 \pm 0.02 \text{ kcal/mol heme}$$

Finally for oxygen binding,

$$Mb + O_2 \rightarrow MbO_2$$

$$\Delta H = -17.4 \pm 0.05 \text{ kcal/mol heme}$$

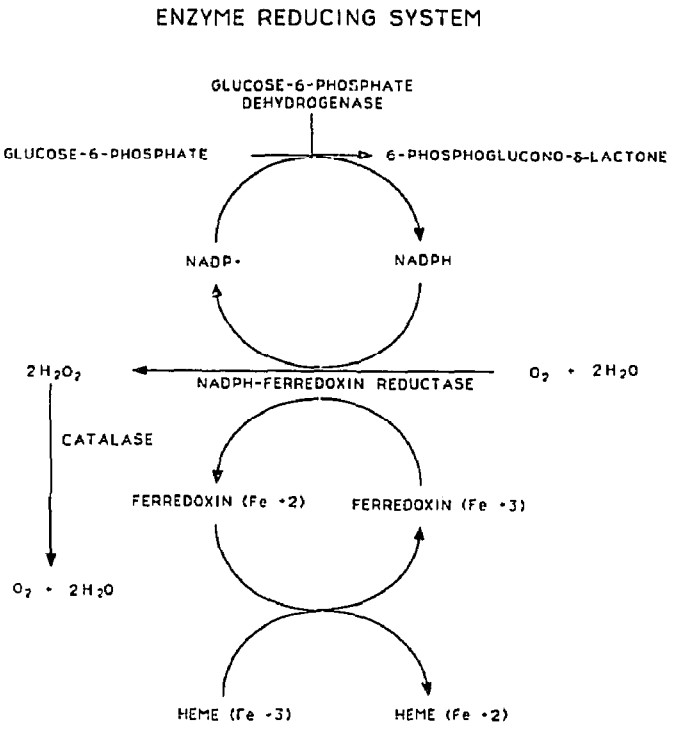


Fig. 3. Enzyme reducing system of Hayashi et al. [1]. Glucose-6-phosphate is not regenerated, therefore an excess must be used.

It was apparent that the enthalpy changes were not internally consistent and it was hypothesized that the enzyme system was interacting with oxygen (Fig. 3). This was tested by reacting the enzyme system, in the absence of myoglobin, with oxygen yielding an exothermic response.

$$ENZ + O_2 \rightarrow ENZ\text{–}O_2?$$

$$\Delta H = -1.41 \pm 0.03 \text{ kcal/mol}$$

(based on heme used)

An exothermic baseline shift in the last two reactions indicated that there was an on-going reaction of the enzyme system with oxygen. The solution to this problem was to place the enzyme system in the reference cell and decrease the concentrations of the components of the system to minimize the effect. Using this method resulted in an oxygen binding enthalpy of

$$Mb + O_2 \rightarrow MbO_2$$

$$\Delta H = -16.0 \pm 0.05 \text{ kcal/mol heme}$$

The reducing system enthalpy of reaction with oxygen is controlled to less than $-0.1$ kcal/mol heme.

The replacement of $O_2$ bound to myoglobin by carbon monoxide was performed under oxygen so that the introduction of a small amount of carbon monoxide did not significantly interrupt the oxygen enzyme reaction (i.e. no baseline shift).

The prospect of oxygen reacting with this commonly used enzyme system leads to the question of whether large amounts of oxygen are being consumed. In some types of oxygen binding experiments this effect could produce significant

Table 2

Experimental conditions

| Species | Buffer | Reducing system |
|---|---|---|
| Sperm whale myoglobin | 2.41 m*M* Heme<br>0.1 *M* Potassium phosphate | 5.0 m*M* Glucose-6-phosphate<br>10 $\mu M$ Catalase |
| Horse myoglobin | 1 m*M* EDTA<br>pH 7.6 | 10 $\mu M$ Ferredoxin<br>100 $\mu M$ NADPH<br>100 $\mu M$ Glucose-6-phophate dehydrogenase<br>1.0 $\mu M$ Ferredoxin–$NADP^+$ reductase |
| Human hemoglobin $A_0$ | 4.19 m*M* Heme<br>0.2 *M* Potassium phosphate<br>2 m*M* EDTA<br>pH 7.6 | 2.0 m*M* Glucose-6-phosphate<br>10 $\mu M$ Catalase<br>10 $\mu M$ Ferredoxin<br>100 $\mu M$ NADPH<br>100 $\mu M$ Glucose-6-phosphate dehydrogenase<br>1.0 $\mu M$ Ferredoxin–$NADP^+$ reductase |

deviations in oxygen activity. Hayashi has suggested that based on the work of Nakamura $H_2O_2$ is being created [1,9]. Ferredoxin–NADP reductase is known to exhibit NADPH oxidase activity in which one mole of oxygen is consumed while NADPH is oxidized to $NADP^+$ (Fig. 3). This process is accompanied by the production of $H_2O_2$. Hayashi noticed that when catalase was excluded from the enzyme system Met hemoglobin production was increased, most likely due to the production of $H_2O_2$ and its oxidative effect on the heme. As Met production is curtailed by the introduction of catalase, it is assumed that the $H_2O_2$ is being changed back into water and oxygen resulting in no net loss of oxygen.

The second problem was discovered when reacting human hemoglobin $A_0$ with carbon monoxide and oxygen under enzymatic conditions. It was found that 50 m$M$ glucose-6-phosphate interacts with hemoglobin to produce larger than expected heats upon ligand addition. To probe this interaction carbon monoxide and oxygen were reacted at 10°C with hemoglobin at 55 and 105 m$M$ glucose-6-phosphate with and without enzymes present (Table 3). At 10°C Met formation is lower allowing for experiments without enzyme reducing system. The reactions were then conducted without enzyme system or glucose-6-phosphate (Table 3).

$$Hb + 4\ CO \rightarrow Hb(CO)_4$$
$$\Delta H = -15.6 \pm 0.05 \text{ kcal/mol heme}$$
$$Hb(O_2)_4 + 4\ CO \rightarrow Hb(CO)_4 + 4\ O_2$$
$$\Delta H = -3.55 \pm 0.02 \text{ kcal/mol heme}$$
$$Hb + 4\ O_2 \rightarrow Hb(O_2)_4$$
$$\Delta H = -12.2 \pm 0.05 \text{ kcal/mol heme}$$

There are two probable explanations for the increase in heat with glucose-6-phosphate. It has been shown that at 50 m$M$ concentrations of glucose-6-phosphate autoglycosylation occurs leading to a shift in the dimer-tetramer equilibrium in the direction of dimer production [10–12]. As noted by Mills and Ackers the enthalpy of binding oxygen to hemoglobin dimers is more exothermic than to the tetrameric form [13]. The second explanation may be that at higher concentrations the phosphate buffer is no longer the dominant species absorbing the released Bohr protons. The heat of protonation for glucose-6-phosphate is potentially more exothermic than phosphate and may result in the appearance of larger heat release [3].

Lower concentrations of glucose-6-phosphate (2 m$M$) yield results similar to those found when the enzyme system is absent, thus reactions were performed under these conditions. Reactions of hemoglobin and reducing enzymes with 2 m$M$ glucose-6-phosphate yield.

$$Hb + 4\ CO \rightarrow Hb(CO)_4$$
$$\Delta H = -15.7 \pm 0.05 \text{ kcal/mol heme}$$
$$Hb(O_2)_4 + 4\ CO \rightarrow Hb(CO)_4 + 4\ O_2$$
$$\Delta H = -3.53 \pm 0.02 \text{ kcal/mol heme}$$
$$Hb + 4\ O_2 \rightarrow Hb(O_2)_4$$
$$\Delta H = -12.2 \pm 0.05 \text{ kcal/mol heme}$$

The enthalpy values for ligand binding to myoglobin were independent of glucose-6-phosphate concentration (0–200 m$M$). Increasing the concentrations of the other enzyme constituents independently did not alter the results for both hemoglobin and myoglobin.

In order to investigate possible total reaction heat capacity changes, a number of experiments

Table 3

Glucose-6-phosphate enthalpy effects (kcal/mole heme)

| Reaction | Glucose-6-phosphate conc. (m$M$) | | | | | |
|---|---|---|---|---|---|---|
| | With enzymes | | | Without enzymes | | |
| | 0 | 55 | 105 | 0 | 55 | 105 |
| $Hb + 4\ CO \rightarrow Hb(CO)_4$ | −15.6 | −16.0 | −18.0 | −15.6 | −16.1 | −17.9 |
| $Hb(O_2)_4 + 4\ CO \rightarrow Hb(CO)_4 + 4\ O_2$ | −3.55 | −3.53 | −3.54 | −3.52 | −3.56 | −3.52 |
| $Hb + 4\ O_2 \rightarrow Hb(O_2)_4$ | −12.2 | −12.5 | −14.5 | −12.2 | −12.4 | −14.6 |

Table 4

$\Delta H$ (kcal/mol heme) for the reaction systems studied as a function of temperature

| Reaction [a] | Temperature (°C) | | | |
|---|---|---|---|---|
| | 10 | 15 | 20 | 25 |
| $M + CO \rightarrow MCO$ [b] | | | | |
| Sperm whale myoglobin | −17.3 | −17.2 | −17.3 | −17.3 |
| Human hemoglobin $A_0$ | −15.7 | −15.6 | −15.6 | −15.6 |
| Horse myoglobin | −16.2 | −16.2 | −16.3 | −16.3 |
| $M + O_2 \rightarrow MO_2$ [b] | | | | |
| Sperm whale myoglobin | −16.0 | −16.0 | −16.0 | −16.1 |
| Human hemoglobin $A_0$ | −12.2 | −12.1 | −12.1 | −12.1 |
| Horse myoglobin | −15.6 | −15.7 | −15.7 | −15.7 |
| $MO_2 + CO \rightarrow MCO + O_2$ [c] | | | | |
| Sperm whale myoglobin | −1.35 | −1.32 | −1.31 | −1.31 |
| Human hemoglobin $A_0$ | −3.53 | −3.54 | −3.54 | −3.53 |
| Horse myoglobin | −0.52 | −0.53 | −0.55 | −0.52 |

[a] Conditions for these reactions are even in Table 2.
[b] Sensitivity: ±0.05 kcal/mol heme.
[c] Sensitivity: ±0.02 kcal/mol heme.

at different temperatures were conducted (Table 4). It is apparent that at this level of precision, the enthalpy change for these reactions over a 15°C temperature range varies less than 0.1 kcal/mol. This is an important observation as one of the basic assumptions in van 't Hoff studies has been that there is little appreciable reactant to product heat capacity effect. The values obtained in this set of experiments compare most favorably with the work of Rudolph et al., Gaud et al., Gill et al. and Imai (Table 1) [15,22,20,21]. The work by the first three groups was done calorimetrically while that done by Imai involved spectral van 't Hoff studies.

In addition to the apparent lack of a reactant to product heat capacity change for ligand binding to hemoglobin and myoglobin there is the observation that the enthalpy change for the oxygen–carbon monoxide replacement reaction of hemoglobin is significantly greater than in myoglobin. Until it is possible to resolve the individual ligand binding enthalpies of hemoglobin, it will not be possible to deduce the origin of this effect.

To further explore the effect of Bohr proton release by hemoglobin seven different sets of buffer conditions were investigated.

(1) 0.2 *M* Potassium phosphate, 2 m*M* EDTA at pH 7.6.
(2) 0.2 *M* Tris-HCl, 2 mM EDTA at pH 7.6.
(3) 0.2 *M* Potassium phosphate, 0.2 *M* NaCl, 2 m*M* EDTA at pH 7.6.
(4) 0.2 *M* Potassium phosphate, 2 m*M* EDTA at pH 6.3.
(5) 0.2 *M* Potassium phosphate, 2 m*M* EDTA at pH 9.0.
(6) 0.2 *M* Tris-$Cl^-$, 2 m*M* EDTA at pH 9.0.
(7) 0.1 M Sodium borate, 2 m*M* EDTA at pH 9.0.

When carbon monoxide or oxygen bind with deoxyhemoglobin at pH 7.6, approximately 2.3 Bohr protons are released [27,7]. As the pH of the solution is increased, there is a corresponding decrease in the Bohr effect; at pH 9.0 the number of protons released nearly vanishes. Similarly as the pH of the solution is decreased from pH 7.6 to 6.3, proton release is again minimal; as the pH is further lowered protons are absorbed upon binding oxygen or carbon monoxide. In order to obtain the intrinsic enthalpy change accompanying ligand binding, the heat of protonation of the buffer must be subtracted from the measured enthalpy. The release of 2.3 protons to the phosphate buffer will contribute −0.5 kcal/mol heme

Table 5a

Enthalpy of binding (kcal/mol heme) at 15.0°C (Solutions contain 1 m*M* EDTA)

| Conditions | Reaction | | |
|---|---|---|---|
| | $Hb + 4\ CO \rightarrow Hb(CO)_4$ | $Hb + 4\ O_2 \rightarrow Hb(O_2)_4$ | $Hb(O_2)_4 + 4\ CO \rightarrow Hb(CO)_4 + 4\ O_2$ |
| 0.2 *M* Potassium phosphate | −15.6 | −12.1 | −3.53 |
| pH 7.6 | −15.1 [a] | −11.6 [a] | |
| 0.2 *M* Tris-HCl | −21.7 | −17.9 | −3.81 |
| pH 7.6 | −15.2 [a] | −11.4 [a] | |
| 0.2 *M* Potassium phosphate | −15.6 | −12.1 | −3.54 |
| 0.2 *M* NaCl | −15.1 [a] | −11.6 [a] | |
| pH 7.6 | | | |

[a] Intrinsic enthalpy assuming 2.3 protons per hemoglobin released upon oxygen or carbon monoxide binding.

to the observed enthalpy [3,7]. Thus the intrinsic enthalpy of CO and $O_2$ binding to hemoglobin at pH 7.6 is −15.1 kcal/mol heme and −11.6 kcal/mol heme respectively. To confirm these values, Tris-HCl buffer was used at pH 7.6 (Condition set 2); its heat of protonation is approximately −11.3 kcal/mol heme. The heat effect of Bohr proton buffer protonation is large and allows for confirmation of the derived intrinsic enthalpy for ligand binding (Table 5a).

It has been demonstrated that the ionic strength and identity of the specific buffer system have significant effects on the free energy of binding carbon monoxide and oxygen to hemoglobin [28,29,7]. In particular phosphate and chloride are known to bind more strongly to the deoxy-hemoglobin state than to the oxy- or carbon-monoxy-state [30,7]. The free energy difference of oxygen binding between buffer systems (1) and (2) is calculated to be less than 0.1 kcal/mol heme [29]. To explore these interactions from an enthalpic perspective sodium chloride was added to the phosphate buffer, yielding buffer condition set (3). There was no detectable change in the enthalpy due to the addition of chloride (Table 5a).

Buffer condition sets (4)–(7) were used to probe the enthalpy change associated with proton release from the hemoglobin molecule (Table 5b). The Bohr effect is known to be small or non-existent at pH 6.3 and 9.0. The data obtained at pH 9.0 is consistent with a Bohr effect of approximately 0.5 protons per hemoglobin molecule without taking into account the binding of the various anions. This suggests that possibly at high anion concentration and pH, the anions bind significantly to both the oxy- and deoxy-forms (i.e. no anion release).

Table 5b

Enthalpy of binding (kcal/mol heme) at 15.0°C (Solutions contain 1 m*M* EDTA)

| Conditions | Reaction | | |
|---|---|---|---|
| | $Hb + 4\ CO \rightarrow Hb(CO)_4$ | $Hb + 4\ O_2 \rightarrow Hb(O_2)_4$ | $Hb(O_2)_4 + 4\ CO \rightarrow Hb(CO)_4 + 4\ O_2$ |
| 0.2 *M* Potassium phosphate pH 6.3 | −19.0 | −15.1 | −3.88 |
| 0.2 *M* Potassium phosphate pH 9.0 | −19.9 | −16.3 | −3.55 |
| 0.2 *M* Tris-HCl pH 9.0 | −21.4 | −17.5 | −3.83 |
| 0.1 *M* Sodium borate pH 9.0 | −20.7 | −17.1 | −3.52 |

In the future we will examine the enthalpy of oxygen binding with hemoglobin and myoglobin as a function of oxygen partial pressure in order to construct oxygen binding curves from which free energy changes for ligand binding can be derived. From these free energy and enthalpy changes a complete picture of the fundamental thermodynamic framework of these reactions should be possible.

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