Calorimetric Studies of Carbon Monoxide and Inositol Hexaphosphate Binding to Hemoglobin A*

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Heats of CO and IHP binding to hemoglobin A have been determined under a variety of buffer and pH conditions. From these data heats of ion binding linked to hemoglobin oxygenation have been estimated.

For IHP binding to deoxyhemoglobin the buffer-corrected enthalpies are surprisingly large, reaching -25 kcal/mol of IHP at pH 7.4. These values correspond to approximately -11 kcal/mol of proton absorbed upon IHP binding and may arise largely from the protonation of histidine and NH₂-terminal groups in the binding site (Arnone, A., and Perutz, M. F. (1974) Nature 249, 34-36). The decreased magnitude of ΔH for IHP observed at low pH parallels the decreased proton uptake at low pH. In 0.1 M chloride (pH 7.4) the reaction

\[ \text{Hb(aq)} + \text{IHP} \rightarrow \text{Hb-IHP}^{\text{aq}} \]

has a standard free energy change (Edalji, R., Benesch, R. E., and Benesch, R. (1976) J. Biol. Chem. 251, 7720-7721) of -10 kcal and an enthalpy change of -25 kcal. Therefore, enthalpic forces provide the dominant driving force of this process. The origin of these large negative enthalpy changes is attributed to the exothermic protonation of protein basic groups induced by the proximity of phosphate negative charges. The importance of protonation in the binding of organic phosphates to hemoglobin may well extend to the specific binding of other phosphate substrates to enzyme reaction sites.

In order to explore the importance of the enthalpy and entropy contributions to the free energy change of ligand-binding reactions, we have directed our attention to the enthalpic components of hemoglobin-ligand reactions. Calorimetric studies of how solution conditions affect heats of hemoglobin-ligand reactions have been particularly limited. Rudolph and Gill (1974) and Nelson et al. (1974) examined pH effects on heats of CO and IHP binding, respectively, to hemoglobin A. Atha and Ackers (1974) evaluated the differential heat of chloride interaction with deoxy and O₂ hemoglobin. Gaud et al. (1975) have attempted to assess buffer effects on the heat of CO binding by HbM Iwate. The experience of these studies shows that a variety of solution conditions such as pH, buffer species, and ionic strength influence the value of the enthalpy change for various ligands. Nonetheless, a comprehensive and self-consistent set of data has been lacking.

We have therefore examined heats of CO and IHP binding to hemoglobin A under a variety of buffer and pH conditions. From these data we are able to estimate and compare the heats of ion binding linked to hemoglobin oxygenation and organic phosphate binding. The results of this study shed interesting light on the sources of the thermodynamic driving force for hemoglobin-allosteric effector interactions. In particular, we find that the enthalpic contribution for binding of IHP to hemoglobin is directly related to proton uptake in the reaction.

MATERIALS AND METHODS

Freshly drawn hemoglobin A was prepared as described by Benesch et al. (1968). The hemoglobin was then either dialyzed against a buffer solution or unbuffered 0.1 M NaCl or, in the case of unbuffered, deionized solutions, it was dialyzed three times against distilled water and then deionized on a Bio-Rex AG 50-X8 mixed bed resin from Bio-Rad Laboratories. The pH was then adjusted with 0.1 M acid or base. The samples were deoxygenated in a tonometer with a cuvette attached and examined spectrally. Details are given by Gaud et al. (1975). The hemoglobin concentration was adjusted to 1 to 2 mM heme. No reducing agents were used since the spectra indicated methemoglobin to be less than 2%. Furthermore, we wished to avoid introduction of any additional ionic species.

The IHP was purchased from P-L Biochemicals as sodium inositol hexaphosphate and analyzed by Huffman Laboratories, Wheatridge, Col., to ascertain water content. Solutions of 25 mM were prepared by weighing the IHP and mixing it with dialysate buffer. The pH was adjusted to that of the original buffer.

The gas-liquid microcalorimeter was described by Rudolph et al. (1972) and improvements are described by Rudolph and Gill (1974) and Gaud et al. (1975). The procedures for liquid titrations and for CO gas titrations are given by Noll et al. (1979). An example of thermal titration with IHP is shown in Fig. 1. Calibrations were performed by reacting CO (g) and 0.1 M HCl with 0.1 M NaOH. The heat values obtained at 25°C were -25.11 ± 0.53 kcal/mol of CO₂ and

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Heat of binding to hemoglobin

-13.4 ± 0.31 kcal/mol of HCl, respectively. These values agree with the literature values for heats of neutralization of CO₂ and of formation of water of -25.70 kcal/mol (Berg, 1975) and -13.34 kcal/mol (Larson and Hepler, 1969), respectively.

RESULTS AND DISCUSSION

Analysis of calorimetric data for CO and IHP binding first requires recognizing that protons released or absorbed in the reactions are abstracted from or absorbed by the buffer with an associated observable heat change. Calculation of this heat effect requires a knowledge of the change in hemoglobin protonation during the reaction. Table I summarizes values effect requires a knowledge of the change in hemoglobin amino~2-(hydroxymethyl)-1,3-propanediol.

\[ H_{b}(IHP)(aq) \]

For any given reaction we found the buffer corrected heats equal within experimental error and the average values for the reactions are summarized in Table II.

The relative pH independence of \( \Delta H_{\text{CO}} \) at about -16.0 kcal/ mol of CO is similar to the -17.5 kcal/mol of CO value observed previously (Rudolph and Gill, 1974) but under different solution conditions. A value of -15.5 kcal/mol of CO is estimated from oxygen-binding heats determined by Atha and Ackers (1974) at pH 7.5. This calculation involves correction for heat of CO solution (-3.0 kcal/mol of CO) and for the heat of replacement of bound O₂ by CO (-4.0 kcal/mol of CO).

For IHP binding to deoxyhemoglobin, the buffer corrected enthalpies are surprisingly large, reaching -25 kcal/mol of IHP at pH 7.4. These values correspond to approximately -11 kcal/mol of proton absorbed upon IHP binding and may arise largely from the protonation of histidine and NH₂-terminal groups in the binding site (Armone and Perutz, 1974). The decreased magnitude of \( \Delta H_{\text{IHP}} \) observed at low pH parallels the decreased proton uptake at low pH. A similar effect is seen for IHP binding to CO-ligated hemoglobin where observed enthalpy changes average about -8 kcal/mol of H⁺ absorbed.

The four reactions constitute a thermodynamic cycle such that \( 4 \Delta H_{\text{CO}} + \Delta H_{\text{IHP}} \) should equal \( 4 \Delta H_{\text{CO}}^{\text{ideal}} + \Delta H_{\text{IHP}} \). The difference is given as \( \Sigma \Delta_{\text{cycle}} \), in the right column of Table II and is well within experimental error.

Differential ion binding by ligated and unligated hemoglobin gives rise to substantial enthalpic effects. For each reaction the buffer correction term was calculated. In the absence of ligand-linked ion binding, all reactions should exhibit ideal enthalpy changes or buffer absorption.

The data show CO-linked chloride ion-binding heats of CO and of formation of water and of formation of water of -25.70 kcal/mol (Berg, 1975) and -13.34 kcal/mol (Larson and Hepler, 1969), respectively.

**Table I**

### Hemoglobin uptake accompanying CO and IHP binding

| pH   | \( \Delta H_{\text{CO}} \) Reaction 1 in text | \( \Delta H_{\text{IHP}} \) Reaction 2 in text | \( \Delta H_{\text{CO}} \) Reaction 3 in text | \( \Delta H_{\text{IHP}} \) Reaction 4 in text |
|------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| 7.4  | -2.0*                                       | -3.1                                       | 2.2                                        | 1.1                                        |
| 7.0  | -1.8                                        | -1.2                                       | 2.1                                        | 2.7                                        |
| 6.5  | -1.2                                        | -0.2                                       | 1.6                                        | 2.6                                        |

* Units of all entries are protons absorbed per Hb tetramer.

**Table II**

### Average buffer-corrected enthalpies of hemoglobin reactions with CO(g) and IHP in 0.1 mM chloride at 25°C

| pH   | \( \Delta H_{\text{CO}} \) Reaction 1 in text | \( \Delta H_{\text{IHP}} \) Reaction 2 in text | \( \Delta H_{\text{CO}} \) Reaction 3 in text | \( \Delta H_{\text{IHP}} \) Reaction 4 in text |
|------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| 7.4  | -16.3 ± 0.3*                                | -11.0 ± 0.4                                | -25 ± 3                                    | -8 ± 4                                     |
| 7.0  | -15.7 ± 0.2                                 | -14.6 ± 1.0                                | -24 ± 2                                    | -22 ± 6                                    |
| 6.5  | -15.5 ± 0.1                                 | -15.2 ± 0.2                                | -19 ± 2                                    | -19 ± 2                                    |

* Values tabulated represent averages of results obtained in Tris and bis-Tris buffers and in unbuffered Hb solutions.
served in chloride solution alone. In 0.1 M chloride containing 0.2 M maleate and 0.2 M phosphate, respectively, we find $\Delta H_{\text{ion}}$ of 4.0 and 4.6 kcal/mol of CO, respectively. In the case of phosphate the same value (4.6 kcal/mol of O$_2$) has been obtained for 0.1 M phosphate containing no chloride by Atha and Ackers (1974). The implication of these results is that, in phosphate-containing solutions, the higher charge may simply contribute to a more exothermic interaction with the deoxy-hemoglobin. This might be due to enhanced protonation of nitrogen groups in the allosteric site as has been suggested for the case of IHP binding (Noll et al., 1979).

The binding of IHP to deoxyhemoglobin must necessarily displace any ions originally bound at the allosteric site. The approach used in analyzing heats of CO-linked ion binding to hemoglobin can be used to estimate thermal effects associated with IHP-linked ion release. The results of this analysis at pH 7.4 are shown in Table IV. The heat of chloride ion binding as replaced by IHP ranges from $-4.1$ to $-8.0$ kcal/mol of IHP in 0.1 M chloride. Tris and bis-Tris give similar values of $-4.1$ and $-4.7$ kcal/mol of IHP, respectively, while in the unbuffered solution yields a value of $-8.0$ kcal/mol of IHP. The higher value observed for the unbuffered case would seem to imply a specific effect of the bis-Tris and Tris buffers on the hemoglobin. On the other hand, since the probable error in these values is in the range of 2 kcal, the differences in the values of $\Delta H_{\text{ion}}$ for the three chloride-containing solutions would barely be significant.

The significantly large heats of $\Delta H_{\text{ion}}$ observed for maleate and phosphate solutions are ascribed to the same factors noted for CO binding, namely increased ion release due to stronger binding and/or higher evolution per ion bound. Both of these factors arise from the higher negative charges of phosphate and maleate which by requiring increased ionic interaction on binding give rise both to higher affinity and more exothermic heats of interaction than is possible for a singly charged species like chloride.

It seems unlikely (Van Beek et al., 1979) for phosphate and maleate that more than two anions are displaced upon IHP binding. If one assumes that two anions are indeed bound in the allosteric site then about 10 kcal are evolved for each anion bound. Heats of such magnitude suggest concomitant protonation as indeed has been observed with the binding of IHP itself.

### Table III

| Solution | $\Delta H_{\text{ion}}$ | $\Delta H_{\text{net}}$ | $\Delta H_{\text{mob}}$ | $\Delta H_{\text{mob}}^*$ |
|----------|------------------------|------------------------|------------------------|------------------------|
| Deionized | $-22.6 \pm 0.6^*$ | $-3.6$ | $-22.6$ | 0 |
| 0.1 NaCl, 0.1 Tris | $-21.6 \pm 0.3$ | $-5.5$ | $-24.6$ | 3.0 |
| 0.1 NaCl | $-19.8 \pm 0.3$ | $-3.6$ | $-22.6$ | 2.8 |
| 0.1 NaCl, 0.2 bis-Tris | $-19.9 \pm 0.3$ | $-3.3$ | $-22.4$ | 2.5 |
| 0.1 NaCl, 0.2 maleate | $-15.8 \pm 0.4$ | $-0.7$ | $-19.8$ | 4.0 |
| 0.1 NaCl, 0.2 phosphate | $-15.0 \pm 0.4$ | $-0.5$ | $-19.6$ | 4.6 |

$^*$ Units of all entries are kilocalories per mol of CO bound.

### Table IV

| Solution | $\Delta H_{\text{ion}}$ | $\Delta H_{\text{net}}$ | $\Delta H_{\text{mob}}$ | $\Delta H_{\text{mob}}^*$ |
|----------|------------------------|------------------------|------------------------|------------------------|
| Deionized | $-14.5 \pm 2.0$ | $-15.8^*$ | $-14.5$ | 0 |
| 0.1 NaCl, 0.1 Tris | $-1.3 \pm 1.0$ | $-24.9$ | $-5.4$ | 4.1 |
| 0.1 NaCl | $-6.5 \pm 1.0$ | $-15.8$ | $-14.5$ | 8.0 |
| 0.1 NaCl, 0.2 bis-Tris | $-10.9 \pm 1.0$ | $-14.7$ | $-27.2$ | 19.4 |
| 0.1 NaCl, 0.2 maleate | $-7.8 \pm 1.0$ | $-3.1$ | $-10.1$ | 21.1 |
| 0.1 NaCl, 0.2 phosphate | $-7.0 \pm 1.0$ | $-2.2$ | $-28.1$ | 21.1 |

$^*$ Units of all entries are kilocalories per mol of IHP bound.

Table V shows the comparison of the differential heats of anion binding to hemoglobin for CO and IHP ligation to deoxyhemoglobin. We note that there is rough agreement between the energetics of ion displacement by the two processes. Such behavior would be expected since on the one hand the binding of IHP to the allosteric site will displace any anions there bound and on the other hand experimental studies of CO binding show that, at least for chloride, both high affinity chloride ions are released. Thus, both CO and IHP ligation of hemoglobin lead essentially to the same anion displacement and should give rise to similar thermal effects.

The magnitude of the thermal effect of IHP binding to deoxyhemoglobin merits further consideration. In 0.1 M chloride (pH 7.4) the reaction

$$\text{Hb}^{aq} + \text{IHP} \rightarrow \text{Hb} \cdot \text{IHP}^{aq}$$

has a standard free energy change (Edalji et al., 1976) of $-10$ kcal and an enthalpy change of $-25$ kcal. Therefore, enthalpic forces provide the dominant driving force of this process. The origin of these large negative enthalpy changes is attributed to the exothermic protonation of protein basic groups induced by the proximity of phosphate negative charges. The importance of protonation in the binding of organic phosphates to hemoglobin may well extend to the specific binding of other phosphate substrates to enzyme reaction sites.

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