Heats of Carbon Monoxide Binding by Hemoglobin M Iwate†

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ABSTRACT: The heat of reaction of CO gas with the $\alpha_2^{Mmet}\beta_2$ and $\alpha_2^{M}\beta_2$ species of the α-chain mutant hemoglobin M Iwate has been studied in buffers with different heats of ionization at 25° and in the absence of organic phosphates. For the $\alpha_2^{Mmet}\beta_2^{deoxy}$ species we find a small Bohr effect (0.12 mol of H^+/mol of CO) which is in correspondence with that found in equilibrium studies. The heat of reaction, when corrected for proton reaction with buffer, is -18.4 ± 0.3 kcal/mol of CO at pH 7.4. At pH 9 the same value is observed within experimental error. This value compares closely with heats of reaction of CO with myoglobin and with van't Hoff determinations of the heat of oxygen binding to isolated hemoglobin α and β chains after correction for the heat of replacement of O_2 by CO. Furthermore, an analysis of the differential heat of ligand binding as a function of the extent of reaction indicated that, within experimental error, the heat of reaction with the first β-chain heme in $\alpha_2^{Mmet}\beta_2^{deoxy}$ is the same as the second. Since the quaternary T→R transition is blocked in this mutant hemoglobin, we compared it with Hb A to estimate the enthalpic component of the allosteric T→R transition in Hb A. The heats of reaction with CO(g) and Hb A are -15.7 ± 0.5 and -20.9 ± 0.5 kcal/mol at pH 7.4 and 9.0, respectively. In going from the T to the R state we find an enthalpy of transition of 9 ± 2.5 kcal at pH 7.4 and -12 ± 2.5 kcal at pH 9.0. From published free energies of transition we conclude the T→R transition is enthalpically controlled at pH 7.4 but entropically controlled at pH 9.0. A near normal Bohr effect is estimated from heats of reaction of CO with $\alpha_2^{Mdeoxy}\beta_2^{deoxy}$ in various buffers. A larger than normal heat of reaction (-21.6 ± 0.5 kcal/mol of CO) is attributed to the abnormal α chains in Hb M Iwate.

In hemoglobin M Iwate a tyrosine replaces the histidine at the F8(87) position of the α chains (Konigsberg and Lehmann, 1965; Shimizu et al., 1965). This critical residue occupies the fifth coordination site of the heme iron. The change of this proximal histidine to tyrosine produces substantial changes in the ligand binding properties of the heme iron (Motokawa et al., 1964; Hayashi et al., 1966, 1967, 1968; Gersonde et al., 1973). In particular, oxygen rapidly oxidizes the α-chain irons of Hb M Iwate to the ferric state which in vivo cannot be reduced by the methemoglobin reductase (Hayashi et al., 1966). In this oxidized state the α-chain iron is coordinated at the sixth position by the distal histidine in the E7(58) position (Greer, 1971). The oxygen carrying capacity is therefore solely due to the two heme groups of the β subunits.

Ligand binding to the β-chain heme groups is significantly modified by the presence of α^{Mmet} chains. The β-chain heme groups are also easily oxidized with oxygen (Gersonde et al., 1973), and thus it is advantageous to use carbon monoxide in studying ligand binding by these two groups. Compared to Hb A, Hb M Iwate ($\alpha_2^{Mmet}\beta_2$) has a much lower CO affinity. The Hill coefficient varies from 1.0 to 1.8 depending on pH, concentration of organic phosphates, and the oxidation state of the neighboring subunits (Gersonde et al., 1973; Sick and Gersonde, 1974). The maximum Bohr effect in stripped $\alpha_2^{Mmet}\beta_2^{deoxy}$ is ~0.14 mol of H^+/mol of CO, much smaller than in Hb A. CO affinity and Bohr effect of the fully reduced Hb M Iwate ($\alpha_2^{Mdeoxy}\beta_2^{deoxy}$) are more nearly normal when compared to Hb A, but oddly the measured Hill coefficient remained near unity (Hayashi et al., 1966).

Crystals of Hb M Iwate have been studied by X-ray crystallographic techniques (Greer, 1971). The lattice parameters of $\alpha_2^{Mmet}\beta_2$ are closely similar to those observed for deoxy Hb A (Muirhead and Greer, 1970). Crystals of this deoxy form do not break up when exposed to air in contrast to the behavior of Hb A (Hüfner, 1884; Nencki and Sieber, 1886; Haurowitz, 1938; Perutz et al., 1964). However, differences between the deoxy and met forms of the β chains in Hb M Iwate were observed. The met form ($\alpha_2^{Mmet}\beta_2^{met}$) has the same quaternary structure as deoxy Hb A. The structural difference between the β deoxy and met forms, as determined directly from the intensity changes of the diffraction patterns, showed that the ligand reaction with the β chain causes widespread changes in its tertiary structure and only a slight movement in the α chain section just across the $\alpha_1\beta_2$ interface. These crystallographic studies lead to the conclusion that Hb M Iwate ($\alpha_2^{Mmet}\beta_2$) exists frozen in the T state with some tertiary structural changes occurring during ligation of the β chains.

The insensitivity of the quaternary structure of the Hb M Iwate to ligation of the β-chain heme irons was also indicated by a study of quaternary structure-sensitive proton nuclear magnetic resonances (Mayer et al., 1973).

We felt that the observed CO binding properties and the structural properties of Hb M Iwate merited an investigation of the thermodynamic changes that occur in ligand binding reactions. Since only two reaction sites of the $\alpha_2^{Mmet}\beta_2$ form are available, this molecule should allow a determination of any difference in the enthalpy associated with the reaction of the first CO molecule as compared with the reaction of the second CO molecule. The determination of the enthalpic changes of the four stepwise reactions in

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Hb A is much more difficult (Gaud et al., 1974; Imai and Tyuma, 1973). Hb M Iwate also affords an opportunity to measure the heat of ligation of hemoglobin β chains whose conformation remains T-like throughout the binding process (see Greer, 1971). Finally, the heat of reaction with the fully reduced form $\alpha_2^{Mdeoxy}\beta_2^{deoxy}$ would indicate what effect the tyrosine replacement at the proximal site has on the enthalpy of the CO binding reaction.

Materials and Methods

The hemoglobin used in this work was Hb M Oldenburg, which has been shown to be identical with Hb M Iwate (Pik and Tönz, 1966). It was prepared according to procedures described elsewhere (Mayer et al., 1973; Gersonde et al., 1973). The hemoglobin M was eluted from carboxymethyl Sephadex C50 with 0.4 *M* NaCl containing 0.02 *M* phosphate (pH 7.8). 2,3-Diphosphoglycerate was removed by passing through Sephadex G50 equilibrated with 0.1 *M* NaCl (Benesch et al., 1968).

The phosphate-free hemoglobin was concentrated to approximately 1 m*M* in heme by ultrafiltration. Samples of approximately 10 ml were dialyzed against 1–2 l. of desired buffers for at least 24 hr. The dialyzed sample in the appropriate buffer was then placed in a special degassing cell with a 1-cm optical cell attached and with the capability of holding an inert atmosphere above the sample. For most purposes an optical spacer was inserted to give a 1-mm light path. The sample was deoxygenated by rotating the optical cuvet so that the sample was continuously smeared over the upper tube cell wall while a stream of inert gas flowed through the cell. Reduction of only the β chains of the Hb M Iwate was accomplished by addition of a fivefold molar excess of ascorbic acid and waiting until a characteristic and stable spectrum between 450 and 640 nm was obtained, usually within 2 hr. Further changes in spectra did not occur during the period of 2–4 days in which the sample was run. After each run the spectrum of the CO-ligated hemoglobin was checked to verify that oxidation had not occurred and that the typical CO spectrum (Motokawa et al., 1964) persisted. Reduction of both the β and the α chains was accomplished by addition of a molar excess of sodium dithionite to the deoxygenated sample at 4° and waiting until the spectrum characteristic of full reduction, similar to that of deoxygenated Hb A, was obtained. Nearly complete reduction was achieved in 3–4 days. The calorimetric behavior (ligand binding capacity and enthalpy) of Hb A is unaffected by such treatment. Moreover, the stability of the Hb M Iwate, in particular, toward dithionite is evidenced by the crystallography of the protein in a 46 m*M* solution of this ion (Greer, 1971). The CO-ligated fully reduced Hb M Iwate gave spectra similar to those observed by Motokawa et al. (1964). In order to test the binding capacity of these reduced hemoglobins, the moles of CO gas reacted were compared with the moles of hemoglobin present as determined either from the hemochrome spectrum (Hayashi et al., 1967; Gersonde et al., 1973) or from the iron concentration measured by atomic absorption. These experiments showed that the ascorbate reduction produced two reactive heme groups, and that the dithionite reduction produced four such heme groups.

Calorimetry. The gas–liquid microcalorimeter used for these measurements has been described previously (Rudolph et al., 1972). As in experiments reported more recently (Rudolph and Gill, 1974), we took special care to equilibrate the reacting gas (CO) with water vapor in the diffusion chamber connected to the calorimeter cell. The CO and argon used were bubbled through alkaline dithionite solution in order to remove traces of oxygen (Sick and Gersonde, 1974). We also tested the gas handling procedures by placing a solution of dithionite into the calorimeter and putting scrubbed CO in the reacting gas diffusion chamber. When the valve between the two chambers was opened, no uptake of gas or heat effect was observed. The calorimeter was operated in either a constant pressure mode where the volume was continually adjusted as the reaction occurred or in a constant volume mode in which the change in pressure was continually followed during the course of the reaction. In the constant pressure mode it is necessary to make a small heat correction for the condensation of water vapor due to the decrease in volume. The heat correction to be subtracted from the observed heat is given by multiplying the fraction of the vapor pressure of water out of the total pressure times the heat of condensation. At 25° this correction was approximately (24/630) × (9.7) = 0.4 kcal/mol of CO reacted. No attempt was made to correct for possible heats of dissolution or for the possible increase in moles of the inert gas due to such dissolution processes. These effects are presumably negligible when virtually all of the diffusing CO gas into the calorimeter reacts with the hemoglobin solution. We have shown in separate experiments that when the diffusion inlet valve is closed while the reaction is in a steady state of gas consumption only a small amount of CO gas continues to react. Near the end of the reaction the buildup of CO partial pressure in the calorimeter might in theory have some error due to heat of CO solution. There were, however, no noticeable differences in experiments where the rates of inlet gas diffusion differed by a factor of two. Experiments run in the constant volume mode do not require the water vapor correction. However, the heat per mole of gas reacted is ΔE since the system is at constant volume. The enthalpy change is given by $\Delta H = \Delta E - fRT$, where ΔE is the heat measured and f is the fraction of total gas volume occupied by the calorimeter. For the system used in these experiments $f = 0.5$ and so the correction fRT is 0.3 kcal/mol. These corrections are near the limits of the experimental errors observed.

An auxiliary electrical heater inserted into the calorimeter was used to check the linearity and accuracy of the calorimetric system. The calibrations showed that quantities of heat observed in the gas reaction experiments could be measured to within 1% accuracy.

The reacting gas is allowed to flow continuously into the calorimeter cell once the reaction begins. The resulting heat effect and electrical compensation are not instantaneous with the observed pressure changes. This lag in time between the measurement of heat and the pressure change is not of any consequence when total heats and total moles of reacting gas are measured, but it is important if we want to compare the heats of reaction per mole of gas reacted along the course of the reaction. In order to bring the heat effect into proper time correspondence with the pressure effect it is necessary to know the time response of the calorimeter to an instantaneous heat burst. The response was found to rise very rapidly and then decay quite slowly with a single exponential with a time constant $\tau = 35$ sec. It can then be shown that an arbitrary input function of heating rate $I(t)$ is simply related to the observed output heating rate $\theta(t)$ by:

$$I(t) = \theta(t) + \tau[d\theta(t)/dt] \quad (1)$$

Thus, the input heating rate caused by the reacting gas can

Table I: Heats of Reaction of CO Gas with Hb M Iwate at 25°.

Buffer (0.2 *M*)	pH	Reduction	Hemoglobin Species	ΔH (kcal/mol of CO)	ΔH_{BC} (kcal/mol of CO)
Sodium maleate	7.4	Ascorbate	$\alpha_2^{Mmet}\beta_2^{deoxy}$	−18.5 ± 0.5	−18.4 ± 0.3[a]
Bis-tris perchlorate	7.4	Ascorbate	$\alpha_2^{Mmet}\beta_2^{deoxy}$	−19.2 ± 0.3	−18.4 ± 0.3
Tris perchlorate	7.4	Ascorbate	$\alpha_2^{Mmet}\beta_2^{deoxy}$	−20.2 ± 0.5	−18.4 ± 0.3
Tris perchlorate	9.0	Ascorbate	$\alpha_2^{Mmet}\beta_2^{deoxy}$	−18.0 ± 0.7	−18.0 ± 0.7
Sodium maleate	7.4	Dithionite	$\alpha_2^{Mdeoxy}\beta_2^{deoxy}$	−21.4 ± 0.5	−21.6 ± 0.5[b]
Bis-tris perchlorate	7.4	Dithionite	$\alpha_2^{Mdeoxy}\beta_2^{deoxy}$	−23.9 ± 0.5	−21.6 ± 0.5

[a] At pH 7.4 the number of Bohr protons released per mole of CO bound to $\alpha_2^{Mmet}\beta_2^{deoxy}$ is calculated to be 0.12 ± 0.02 while at pH 9.0 it is assumed to be 0. [b] At pH 7.4 the number of Bohr protons released per mole of CO bound to $\alpha_2^{Mdeoxy}\beta_2^{deoxy}$ is calculated to be 0.3 ± 0.1

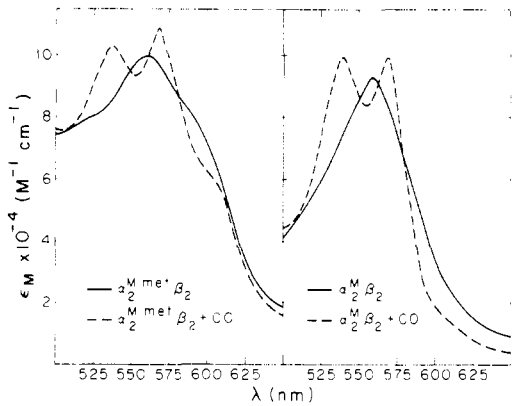

FIGURE 1: Spectra of deliganded and CO-binding hemoglobin M Iwate in the $\alpha_2^M\beta_2$ and $\alpha_2^{Mmet}\beta_2$ forms at 25° in 0.2 *M* bis-tris (pH 7.4). Measurements were performed on solutions ~0.7 m*M* in heme as determined by atomic absorption.

be determined from the output heating rate as a function of time. Also note that the total heat effect given by integration of eq 1 from an initial to a final state where $d\theta(t)/dt$ equals zero is given either by the time integral of $I(t)$ or that of $\theta(t)$. This deconvolution of $\theta(t)$ to $I(t)$ was applied to several runs where sufficiently many values of $\theta(t)$ were available. A least-squares polynomial fit was used to represent $\theta(t)$ from which first the derivative and then $I(t)$ were determined. From these values the amount of heat could be determined for a particular time interval of a measured pressure change (or moles of gas reacted). In this way a table of heats per mole of gas reacted could be generated from the experimental data.

Data Analysis

We require the relation of the Hill coefficient to the equilibrium constants for a two-step reaction. The ligand binding reactions are:

$$M(aq) + CO(g) \longrightarrow M(CO)(aq) \qquad K_1,\ \Delta H_1 \quad (2)$$

$$M(CO)(aq) + CO(g) \longrightarrow M(CO)_2(aq) \qquad K_2,\ \Delta H_2 \quad (3)$$

where K_1 and K_2 are the appropriate equilibrium constants and ΔH_1 and ΔH_2 are the heats of reaction for the defined reactions. The average number $\bar{n}$ of ligands bound per mole of protein is given by:

$$\bar{n} = \frac{K_1 p_{CO} + 2K_1K_2 p_{CO}^2}{1 + K_1 p_{CO} + K_1K_2 p_{CO}^2} \quad (4)$$

The Hill coefficient, n_H, is then defined as:

$$n_H = \frac{d \ln [\bar{n}/(2 - \bar{n})]}{d \ln p_{CO}} \quad (5)$$

For half-saturation we find that the Hill coefficient determines the ratio K_2/K_1 by the relation shown in eq 6. Thus,

$$K_2/K_1 = n_H^2/4(2 - n_H)^2 \quad (6)$$

where the Hill coefficient is known for a two-step reaction it is a simple matter to calculate the ratio of equilibrium constants.

At any point in the reaction, the total heat evolution q up to that point is simply:

$$q = n_{Hb}[\Delta H_1(\alpha_1 + \alpha_2) + \Delta H_2\alpha_2] \quad (7)$$

where α_1 and α_2 are the fractions of the total moles, n_{Hb}, of hemoglobin in the mono- and diliganded states, respectively. Differentiation with respect to n, the total moles of ligand bound, yields eq 8, where dq/dn is the differential

$$\frac{dq}{dn} = \Delta H_1\left(\frac{\partial \alpha_1}{\partial \bar{n}} + \frac{\partial \alpha_2}{\partial \bar{n}}\right) + \Delta H_2 \frac{\partial \alpha_2}{\partial \bar{n}} \quad (8)$$

heat of ligand binding and is the quantity we measure as a function of $\bar{n}$ in our experiments.

Since we can assume precise knowledge of ΔH_T as q_{total}/n_{total}, we can eliminate one parameter:

$$\Delta H_T = \Delta H_1 + \Delta H_2 \quad (9)$$

$$\left(\frac{dq}{dn} - \Delta H_T \frac{\partial \alpha_2}{\partial \bar{n}}\right) = \Delta H_1 \frac{\partial \alpha_1}{\partial \bar{n}} \quad (10)$$

So, by the least-squares condition:

$$\Delta H_1 = \frac{\sum_{pts}\left(\frac{dq}{dn} - \Delta H_T \frac{\partial \alpha_2}{\partial \bar{n}}\right)\left(\frac{\partial \alpha_1}{\partial \bar{n}}\right)}{\sum_{pts}(\partial \alpha_1/\partial \bar{n})^2} \quad (11)$$

$$\Delta H_2 = \Delta H_T - \Delta H_1 \quad (12)$$

If we calculate σ, the standard deviation of dq/dn about the fitted curve, then the standard deviations $\sigma_{\Delta H_1}$ and $\sigma_{\Delta H_2}$ of the fitted constants are given as shown in eq 13. Since

$$\sigma_{\Delta H_1} = \sigma_{\Delta H_2} = \sigma \frac{1}{\Sigma(\partial \alpha_1/\partial \bar{n})^2} \quad (13)$$

$\partial\alpha_1/\partial\bar{n}$ and $\partial\alpha_2/\partial\bar{n}$ are most easily calculated in terms of p_{CO}, it is necessary to solve eq 4 for p_{CO} at each point in the reaction.

Results

The optical spectra of the various samples studied are shown in Figure 1. The $\alpha_2^{Mmet}\beta_2^{deoxy}$ spectrum exhibits, because of the presence of α^{Mmet} subunits, a shoulder at 615 nm characteristic of methemoglobin (Motokawa et al., 1964; Gersonde et al., 1973). Dithionite treatment causes complete reduction to the $\alpha_2^{Mdeoxy}\beta_2^{deoxy}$ state. The spectra

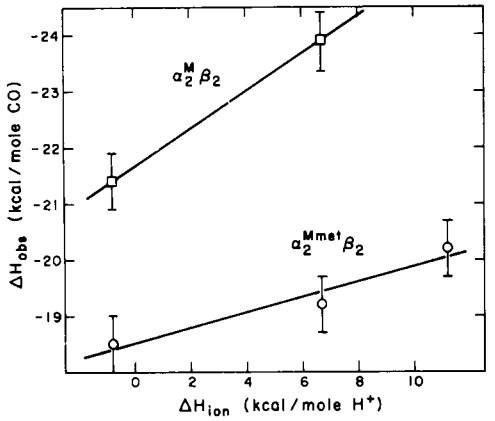

FIGURE 2: Plots of observed heats ΔH_{obsd} of CO binding vs. heats ΔH_{ion} of buffer ionization for hemoglobin M Iwate in the $\alpha_2^M\beta_2$ and $\alpha_2^{Mmet}\beta_2$ forms. Measurements were performed at 25° and pH 7.4 in buffers described in the text.

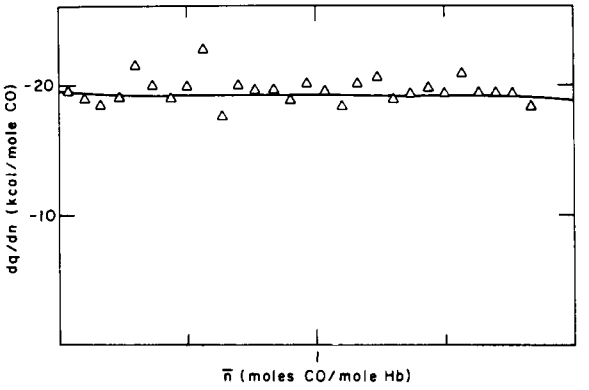

FIGURE 3: Plot of differential heat $dq/d\bar{n}$ of CO binding by the $\alpha_2^{Mmet}\beta_2$ form of hemoglobin M Iwate as a function of the average number $\bar{n}$ of moles of CO bound per mole of hemoglobin. Measurements were performed at 25° and pH 7.4 in 0.2 *M* bis-tris perchlorate buffer. The sample was 6 ml of solution ~1.7 m*M* in heme.

of this species, both ligand free and with CO bound, are very similar to the corresponding spectra for Hb A.

The results of the calorimetric experiments are summarized in Table I. In the first set of experiments Hb M Iwate as $\alpha_2^{Mmet}\beta_2^{deoxy}$ produced by ascorbate treatment was allowed to react with CO(g). The enthalpy of reaction, ΔH, per mole of CO(g) represents the total heat evolved divided by the number of moles of CO allowed to react. The reproducibility of duplicate experiments was ±3%. Where only a single experimental value could be trusted we give an approximate estimate of the uncertainty of the enthalpy of reaction. The various buffer conditions were chosen to permit us to measure the magnitude of the Bohr effect. These measurements are totally independent of previous Bohr effect determinations from pH titration or from pH dependence of binding isotherms (Hayashi et al., 1966; Gersonde et al., 1973). We attribute the different heats of reaction observed in the different buffers to the heat of reaction of the released Bohr protons with the different buffers. The heats of ionization of 25° for maleate, bis-tris,[1] and Tris were taken to be −0.8, 6.7, and 11.2 kcal/mol, respectively (Sober et al., 1970; Paabo and Bates, 1970). A plot of $-\Delta H$ vs. the heats of buffer ionization (ΔH_{ion}) is shown in Figure 2. Within experimental error the results are fitted by a straight line. We describe this line by eq 14, where ΔH_{BC} is

$$-\Delta H = -\Delta H_{BC} + (n_{H^+})\Delta H_{ion} \quad (14)$$

the buffer-corrected value representing the heat of ligand binding corrected for the heat of Bohr proton uptake by the buffer, and n_{H^+} is the number of Bohr protons released per mole of CO allowed to react (Rudolph and Gill, 1974). We have often used this equation to correct ΔH for heat of proton uptake by buffer, using independently known values of Bohr proton release. In such cases we have always obtained a single value for ΔH_{BC}, whatever the buffer ions present. For example Hb A + CO yields the same ΔH_{BC} in sodium maleate, bis-tris chloride, and bis-tris perchlorate at pH 7.4. While it is known that chloride ion for example exhibits differential binding to oxy- and deoxy-HbA (Chiancone et al., 1972), we find *no* thermal effects attributable to such binding either of chloride or of any other ion used in our buffer systems. Fitting eq 14 to our data gives $\Delta H_{BC} = -18.4 \pm 0.3$ kcal/mol of CO and $n_{H^+} = 0.12 \pm 0.02$ mol of H^+ released/mol of CO. We felt that measuring the heat of reaction at a pH where the Bohr effect is practically absent would provide an additional check on the value of ΔH_{BC}. The heat of CO binding at pH 9 represents the intrinsic heat of reaction ΔH_{int} while ΔH_{BC} is the sum of ΔH_{int} and the heat of Bohr proton release. One run at pH 9.0 in Tris-ClO_4^- gave a value of ΔH_{int} close to the value of ΔH_{BC} found at pH 7.4.

[1] Abbreviation used is: bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

Table II: Heats of Reaction of CO Gas with Hemoglobin A at 25°.

Buffer (0.2 *M*)	pH	ΔH (kcal/mol of CO)	ΔH_{BC}[a] (kcal/mol of CO)
Sodium maleate	7.4	−15.3 ± 0.5	−15.7 ± 0.5
Bis-tris perchlorate	7.4	−19.1 ± 0.5	−15.7 ± 0.5
Tris perchlorate	7.4	−21.5 ± 0.5	−15.7 ± 0.5
Tris perchlorate	9.0	−21.8 ± 0.5	−20.9 ± 0.5

[a] At pH 7.4 the number of Bohr protons released per mole of CO bound to $\alpha_2\beta_2$ is calculated to be 0.51 ± 0.05 while at pH 9.0 it is taken to be 0.08 (Antonini et al., 1965).

Unfortunately our measurements do not have sufficient precision to calculate the heat of ionization ΔH_{Bohr} of the Bohr group which could be estimated as shown in eq 15.

$$\Delta H_{Bohr} = [\Delta H_{BC}(\text{pH } 7) - \Delta H_{int}]/n_{H^+}(\text{pH } 7) \quad (15)$$

The most precise measurements were obtained on the runs with the bis-tris buffer. The results of a representative experiment are shown in Figure 3; other $\alpha_2^{Mmet}\beta_2$ runs gave similar plots. Values of the average differential heat of CO binding corrected for calorimeter time response using eq 1 are plotted vs. the average number $\bar{n}$ of CO molecules bound per mole of hemoglobin.

Three samples were treated with dithionite. Two of these were run in bis-tris perchlorate and one in sodium maleate buffer. The quantity of CO gas which reacted corresponded to 1 mol of CO per mol of heme. Compared to the ascorbate reduced samples, the reaction was considerably more exothermic and the Bohr effect more pronounced, as shown by the results in Figure 2. For this material we find $\Delta H_{BC} = -21.6 \pm 0.5$ kcal/mol of CO and $n_{H^+} = 0.3 \pm 0.14$.

Measurements were also made on Hb A under similar experimental conditions. These values are given in Table II.

Discussion

The measurements of the heats of reaction of stripped

$\alpha_2^{Mmet}\beta_2^{deoxy}$ with CO in various buffers at pH 7.4 confirm the presence of the small Bohr effect $n_{H^+} = 0.15$ noted by other work on the pH dependence of log $p_{CO(1/2)}$ (Gersonde et al., 1973).

Since the results in Figure 2 are adequately expressed by a simple linear relationship it appears unlikely that there are substantial thermal effects due to differential buffer interaction with the unliganded and liganded proteins. The intrinsic heat of reaction which excludes the heat of Bohr ionization is suggested as -18.4 ± 0.6 kcal/mol. It is interesting to compare this value with determinations of the heats of reaction on myoglobin and separated hemoglobin α and β chains where Bohr effects are known to be absent. A value of -18.1 ± 0.3 kcal/mol was obtained for the reaction of horse myoglobin with CO(g) (Rudolph et al., 1972). De Renzo et al. (1967) have reported a value of -13.5 kcal/mol of O_2 as determined by the temperature dependence of the equilibrium constant for reaction of O_2(g) with either separate α- or β-chain solutions. The heat of replacement of O_2 with CO has been reported by Roughton (1954) as -5.0 kcal/mol or in more recent studies as -4.0 kcal/mol (Gaud et al., 1974). Thus, we estimate that the heats of CO(g) reacting with α and β chains will be -17.5 ± 1.0 kcal/mol of CO. Since all of these values are in close agreement, there is a strong implication that the value of -18.0 kcal represents the heat of the CO–heme reaction in the absence of quaternary structural changes.

Further evidence for this conclusion comes from the examination of the heat of reaction as a function of extent of reaction. In Figure 3 we have given the results for the CO reaction with $\alpha_2^{Mmet}\beta_2^{deoxy}$ at pH 7.4 in bis-tris buffer. The differential heat of CO binding appears independent of the average number $\bar{n}$ of CO bound per Hb. The limiting values of $dq/d\bar{n}$ at $\bar{n} = 0$ and $\bar{n} = 2$ represent ΔH_1 and ΔH_2. While the accuracy of the data below 5% and above 95% saturation does not justify direct extrapolation of $dq/d\bar{n}$ to the ΔH_1 and ΔH_2 values, the entire data set can be treated with the least-squares procedure discussed under Materials and Methods. A value of $K_2/K_1 = 4$ was calculated by eq 6 from the Hill coefficient $n_H = 1.6$, determined by Gersonde et al. (1973) for stripped $\alpha_2^{Mmet}\beta_2^{deoxy}$ at pH 7.4. Solution of eq 11 and 12 for the data in Figure 3 gives $\Delta H_1 = -19.6 \pm 0.9$ and $\Delta H_2 = -18.7 \pm 0.9$ kcal/mol of CO. The standard deviation of this data set is 1.08 kcal/mol of CO. We therefore conclude that, within experimental error, the heat of reaction is the same for the first and second steps of CO binding to $\alpha_2^{Mmet}\beta_2^{deoxy}$. This implies that the source of the cooperative value of K_2/K_1 is due to an entropic difference in the binding of the first and second ligands. In the absence of cooperativity $K_2/K_1 = 1/4$ and thus the cooperativity represented by $K_2/K_1 = 4$ is a factor of 16 which would be equivalent to 5.5 entropy units, or 1.6 kcal for $T\Delta S$ at 25°.

It therefore appears that a value of -18.0 ± 0.6 kcal/mol of CO is independent of whether the heme group is present in an isolated hemoprotein chain such as myoglobin or hemoglobin α chains, or whether it is part of tetrameric structure such as $\alpha_2^{Mmet}\beta_2$ in which the quaternary structure apparently remains unchanged[2] during the ligation reaction (Greer, 1971; Perutz, 1972). This value is apparently insensitive to the pH as seen in our measurements on the Hb M Iwate $\alpha_2^{Mmet}\beta_2^{deoxy}$ and the absence of Bohr effects in single chain species. We can therefore assess the contribution that the allosteric transition from the quaternary T to R structure makes to the heat of the reaction of normal Hb A. For the purpose of this assessment we report measurements of the CO(g) reaction with Hb A at pH 7.4 and 9.0 in several different buffers. The heat of reaction of Bohr protons released with the buffers involved are subtracted to give ΔH_{BC} as shown in Table II. These values agree within experimental error with determinations made by Atha and Ackers (1974) provided one corrects for the difference of the heats of solution of O_2 and the replacement heat of CO for O_2 in hemoglobin (-4.0 kcal/mol). Furthermore, the value of -15.7 kcal/mol of CO agrees with the results found by Imai and Tyuma (1973) who determined the average heat of reaction from the temperature dependence of the median partial pressure of O_2 gas reaction with Hb A as -11.7 ± 0.6 kcal/mol of O_2.

Table III: Thermodynamics of the T→R Allosteric Transition in Hemoglobin A at 25°.

	pH 7.4	pH 9
$\Delta G_{T\to R}$ (kcal/mol of Hb)[a]	+4.7	+4.0
$\Delta H_{T\to R}$ (kcal/mol of Hb)[b]	+9 ± 2.5	12 ± 2.5
$T\Delta S_{T\to R}$ (kcal/mol of Hb)[c]	+4.3 ± 2.4	16 ± 2.4

[a] Tyuma et al. (1973). Calculated as $\Delta G_{T\to R} = -RT \ln [(k_1/k_4)\cdot(k_2/k_4)(k_3/k_4)]$. [b] Calculated as $4[\Delta H_{BC}(\frac{1}{4}\text{Hb A} + \text{CO}) - (18.0 \pm 0.6)]$ kcal/mol. [c] Calculated as $\Delta H_{T\to R} - \Delta G_{T\to R}$.

In Table III we show the results of calculating thermodynamic properties of the T to R allosteric transition in Hb A. We have based the calculation of $\Delta H_{T\to R}$ on the assumption that the difference in the observed buffer corrected heat of reaction of Hb A with CO(g) at different pH values minus the heat of reaction of Hb M Iwate ($\alpha_2^{Mmet}\beta_2^{deoxy}$) buffer corrected gives a measurement of the heat of the allosteric T→R transition. Since 4 mol of CO is involved in the transition process this factor is multiplied times the difference of appropriate values listed in Tables I and II. The values of the free energy attributed to the allosteric transition are calculated from successive step determinations by Tyuma et al. (1973) on bis-tris solutions at pH 7.4 and on glycine-buffered solutions at pH 9.1. We assume the intrinsic free-energy change of the fourth step is equivalent to that of an isolated chain as shown by Tyuma et al. (1971). Thus, the contribution of the allosteric transition is calculated from the differences between the first three steps and the last. The calorimetric determination of $\Delta H_{T\to R} = 9 \pm 2$ kcal/mol agrees with the value derived by Imai and Tyuma (1973) from the temperature dependence of the T to R equilibrium constant for Hb A at pH 7.4 in 0.1 *M* NaCl. This general agreement of the heat of transition by two entirely different methods is reassuring. Since the estimation of our $\Delta G_{T\to R}$ is based on a different set of assumptions than those used by Imai and Tyuma (1973) we obtain somewhat different values, though still positive for $\Delta S_{T\to R}$. We note the importance of the enthalpic term as the principal determinant of the free-energy change under normal pH conditions. The higher enthalpy observed for the T state is consistent with the terminology of this being a "tense" conformation (Perutz, 1970). The situation observed at pH 9 is markedly different. Here we find an exothermic heat of transition, although the allosteric free energy for the transi-

[2] Ligation of β_4 has definitely been shown to take place without conformational change. Calorimetric measurements on this material would thus provide another check on our hypothesis and we are currently arranging a source of HbH so that these experiments can be performed.

tion process is quite similar to that observed at pH 7.4. A direct consequence of this situation is seen in the large negative entropy change at pH 9. Thus, at high pH values where the Bohr effect is absent and does not make an endothermic contribution to the heat of transition, we find that the T state must be stabilized by a significant entropic term. The molecular interpretation of the exothermic heat of transition and the negative entropy change at high pH may be due to (1) the transfer of a charged group originally embedded in the T structure to a water-exposed position in the R state, or (2) the exposure to solvent of more hydrophobic groups in the R state than in the T state causing the increased formation of structured water with a consequent exothermic heat effect. The latter suggestion is supported by some unpublished observations of a relatively large positive heat capacity change for the reaction of Hb A and CO(g) corrected for the Bohr effect.

That Hb M Iwate is highly cooperative in ligand binding (n = 1.6) while no gross T→R transition is observed suggests that hemoglobin structural changes on ligation occur on two scales. The gross rearrangement of subunits one to another in Hb A both changes the environments, and hence the pK values, of surface groups (Bohr effect) and necessitates an alteration of crystal lattice parameters. This large-scale motion is probably the cause of transition enthalpies we calculate. On the other hand cooperativity itself minimally requires only subtle molecular motions and, as Hb M Iwate makes clear, can take place without grossly observable quaternary structure changes.

The heat of reaction observed for the fully reduced form of Hb M Iwate is nearly 6 kcal/mol of CO more exothermic than that observed for Hb A at pH 7.4. This large difference is presumably due to the replacement of tyrosine for the proximal histidine in the α chains of Hb Iwate. The increased exothermicity for the CO binding reaction perhaps is indicative of greater reactivity of these abnormal heme groups and consistent with their facile conversion to met heme in the presence of oxygen.

The results of this study of Hb M Iwate suggest that the enthalpic changes for the T to R transition can be studied by comparing Hb A with other systems where the transition is either chemically restricted or, as for example in the β_4 molecule, inherently absent.

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