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THERMODYNAMIC ANALYSIS OF CARBON MONOXIDE BINDING BY HEMOGLOBIN TROUT I ☆

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Calorimetric measurements at 25° of the differential heat of CO binding by hemoglobin trout I have been examined together with the CO binding isotherms for the protein at 4° and 20°. Simultaneous treatment of these data sets by a statistically rigorous technique permits evaluation of all the thermodynamic parameters for both the Adair and the Monod, Wyman, Changeux (MWC) models. The results show the details of the unusual temperature dependent cooperativity which this hemoglobin exhibits. In the Adair formalism the increasingly favorable free energy change for successive steps of ligand binding are nearly linearly paralleled by increasingly negative enthalpy changes for these steps. This causes the enhanced cooperativity observed as the temperature is decreased. For the MWC case, lowering the temperature increases the stability of the unligated T state relative to the unligated R state since the enthalpy of the T → R transition is 29.4 kcal mol^{-1}. Simultaneously, the favorability of ligating R forms relative to T is enhanced since R form ligation is 14.1 kcal $(mol\ CC)^{-1}$ more exothermic than that of T. The balance between these opposing effects is to increase ligand binding cooperativity at low temperatures. The predicted temperature dependence of the Hill coefficient for the MWC and Adair models is identical at low and intermediate temperatures but, interestingly, would show a strong divergence at high temperatures where negative cooperativity is suggested for the Adair case and positive cooperativity for the MWC case.

1. Introduction

Our interest in establishing thermodynamic parameters for ligation of hemoglobin trout I stems from two perspectives. First, we desired to know how the thermodynamic parameters for stepwise CO-ligation of this interesting hemoglobin compared to the parameters assignable [1] on the basis of the concerted MWC ‡ model. Second, we have for some time been interested in learning precisely what types of data are necessary unambiguously to assign all the modynamic parameters to the steps of multi-step processes such as gas binding by hemoglobins. The results of our early attempts were not totally satisfactory [2,3].

These studies make clear that calorimetric data alone are insufficient to establish stepwise enthalpy changes for cooperative multi-step processes. On the other hand, to evaluate such heats by van 't Hoff techniques from equilibrium constants requires a data precision not always attainable [4,5]. We concluded, therefore, that simultaneous measurements both of ligand binding isotherms and of differential heats of ligation would enable reaction heats and free energies to be calculated with optimal precision. We used this procedure in our studies of hemoglobin M Iwate

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‡ Abbreviations used in this paper are: MWC, Monod, Wyman Changeux; bis-tris, bis-(2-hydroxyethyl)-imino-tris-(hydroxymethyl) methane; P_{50}, ligand partial pressure at 50% saturation of protein.

where two sites only are available for ligand binding and, hence, where both equilibrium constants can be evaluated from measurements of p_{50} and the slope of the Hill plot [6].

For four-site hemoglobins of reasonably high cooperativity, stepwise binding constants are difficult to determine with precision from binding isotherms and stepwise heats determined from calorimetry depend critically on these binding constants. We ultimately concluded that some method would have to be found whereby the temperature variation of isotherms could be constrained to represent the same van 't Hoff stepwise heats as those directly suggested by calorimetry. Also and conversely, the shape of plot of differential heat of ligand binding versus extent of reaction at any temperature must be consistent with the equilibrium constants suggested by the binding isotherm at that temperature. How binding equilibrium and calorimetric data sets obtained at different temperatures may be simultaneously processed to yield a single set of stepwise thermodynamic parameters is the subject of this communication.

2. Experimental

Hemoglobin-CO binding isotherms were measured in 0.1 M sodium phosphate buffer at pH 6.8 [1,7]. Deoxyhemoglobin solutions (0.25–1 μM heme) containing a slight excess of sodium dithionite were titrated with solutions of known CO concentration ($\sim 1 \times 10^{-4}$ M) [7]. Titrations were performed in the dark in cells of 5 cm light path and 26 ml total volume, no gas space being present. Extent of ligation was determined using a Cary 14 spectrophotometer by difference spectra in the 400–450 nm region. Hemoglobin concentrations were determined spectrophotometrically assuming $\epsilon_{542}^{mM} = 12\,300$ cm^{-1} for the fully oxygenated protein. Identical results were obtained for heme concentrations of 0.25–1.0 μM. This, taken together with the known very small value of the dimer-tetramer dissociation constant [8], suggests that the presence of dimers can be neglected.

Calorimetric measurement of differential heats of CO binding to the hemoglobin were performed generally as previously described [6,9]. A sensitive gas-liquid microcalorimeter [10] yielded the heat of reaction as a function of gas uptake. The extent of reaction was evaluated from changes in system pressure at constant volume, these changes being followed by a Validyne Company differential manometer. Enthalpy changes were obtained from the internal energy values initially obtained by subtraction of the PV term and were corrected for the –2.9 kcal (mol CO)$^{-1}$ heat of solution of CO. Experiments were performed at 25° in buffers of 0.2 M sodium maleate, pH 7.1, and in 0.05 M bis-tris chloride, pH 7.4. Deoxyhemoglobin solutions were nominally 1 mM heme.

3. Data analysis

Suppose that we have for various temperatures two types of thermodynamic data sets, fractional saturation θ of the protein as a function of free ligand concentration x and differential heat of ligand binding $dq/d\bar{n}$ as a function of the average number $\bar{n}$ of moles of ligand bound per mole of protein. If we assume that these equilibria obey the Adair [11] scheme,

$$\mathrm{HbX}_{i-1} + \mathrm{X} \xrightarrow[\Delta H_i]{K_i} \mathrm{HbX}_i \quad (i = 1 \text{ to } 4). \tag{1}$$

Then the experimental isotherm data should obey the equation

$$\theta = \frac{1}{4}\frac{\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3 + 4\beta_4 x^4}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4}, \tag{2}$$

where

$$\beta_i = \prod_{j=1}^{i} K_j. \tag{3}$$

Alternatively in the MWC model,

$$\begin{array}{ccc} \mathrm{Hb}^{\mathrm{T}} & \xrightarrow[\Delta H_{\mathrm{T}}]{k_{\mathrm{T}}} & \mathrm{Hb}^{\mathrm{T}}\mathrm{X} \rightarrow \ldots \rightarrow \mathrm{Hb}^{\mathrm{T}}\mathrm{X}_4 \\ L \uparrow\downarrow & \Delta H_L & \\ \mathrm{Hb}^{\mathrm{R}} & \xrightarrow[\Delta H_{\mathrm{R}}]{k_{\mathrm{R}}} & \mathrm{Hb}^{\mathrm{R}}\mathrm{X} \rightarrow \ldots \rightarrow \mathrm{Hb}^{\mathrm{R}}\mathrm{X}_4 \end{array} \tag{4}$$

The isotherm becomes

$$\theta = \frac{1}{4}\frac{4k_T x(1+k_T x)^3 + 4Lk_R x(1+k_R x)^3}{(1+k_T x)^4 + L(1+k_R x)^4}. \quad (5)$$

It is convenient, for future purposes, to define derivatives of the binding polynomials [12] of the models. For the Adair scheme, the binding polynomial is given by

$$P_A = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4. \quad (6)$$

For the MWC model

$$P_M = (1+L)^{-1}[(1+k_T x)^4 + L(1+k_R x)^4]. \quad (7)$$

In both cases, the following derivatives of the polynomials are defined

$$P^{(1)} = \partial P/\partial \ln x, \qquad P^{(2)} = \partial^2 P/\partial(\ln x)^2. \quad (8)$$

In the particular experiments performed here the free ligand x is not measured directly but rather is calculated from the total ligand x_t by use of the relation

$$x = x_t - 4\theta[\mathrm{Hb}]_t \quad (9)$$

where $[\mathrm{Hb}]_t$ is the total concentration of hemoblogin tetramers.

The dependent variable θ in the saturation experiments is thus defined in terms of the independent variable x_t by eliminating x between equations (9) and (2) or (5). The calorimetric data is not quite so simply represented. In the Adair case if we let α_i represent the fraction of protein present as HbX_i, then we define the quantities f_i by

$$f_i = \sum_{j=1}^{5-i} \alpha_{5-j}. \quad (10)$$

With this assignation, the differential heat of ligand binding at any point in the reaction may be written

$$\frac{dq}{d\bar{n}} = \Delta H_1 \frac{df_1}{d\bar{n}} + \Delta H_2 \frac{df_2}{d\bar{n}} + \Delta H_3 \frac{df_3}{d\bar{n}} + \Delta H_4 \frac{df_4}{d\bar{n}}. \quad (11)$$

The derivatives are easily evaluated from the expressions from the α_i by the chain rule:

$$\frac{df_i}{d\bar{n}} = \sum_{j=1}^{5-i} \frac{\beta_j x^j (jP_A - P_A^{(1)})}{P_A P_A^{(2)} - P_A^{(1)} P_A^{(1)}}. \quad (12)$$

For the MWC case we define the quantities ϕ_L, ϕ_T, and ϕ_R as the fractions of all sites present on R-conformation molecules, of ligated sites on T-conformation tetramers, and of ligated sites on R-conformation tetramers, respectively. Then

$$\phi_L = L(1+k_R x)^4/P_M, \quad (13)$$

$$\phi_T = 3k_T x(1+k_T x)^3/P_M, \quad (14)$$

$$\phi_R = 3Lk_R x(1+k_R x)^3/P_M. \quad (15)$$

Differentiating with respect to $\bar{n}$ to obtain the differential heat of CO binding, there results

$$\frac{dq}{d\bar{n}} = \Delta H_L \frac{d\phi_L}{d\bar{n}} + \Delta H_T \frac{d\phi_T}{d\bar{n}} + \Delta H_R \frac{d\phi_R}{d\bar{n}}. \quad (16)$$

wherein the derivatives are

$$\frac{d\phi}{d\bar{n}} = \frac{(d\phi/dx)P_M^2}{P_M P_M^{(2)} - P_M^{(1)} P_M^{(1)}}, \qquad \phi = \phi_L, \phi_R, \phi_T. \quad (17)$$

Since $\bar{n} = 4\theta$, the dependent variable $dq/d\bar{n}$ of the calorimetric experiments is defined in terms of the independent variable $\bar{n}$ by eliminating x between equations (2) and (11) or between (5) and (16), respectively.

In processing the data our goal is to obtain at some reference temperature, say 25°C, the stepwise binding constants and the stepwise binding enthalpies which would have the highest probability of yielding the observed data sets. We make the assumption that, in the binding curve experiments, the standard errors σ_θ in θ are much larger than those in x_t; moreover, we assume them to be normally distributed. This first assumption is supported by observation and the second results from θ being calculated for the small difference in two large quantities, namely absorbances. Likewise we assume that, in the calorimetric data, the errors in $dq/d\bar{n}$ overpower those in $\bar{n}$, are independent of $\bar{n}$, and are normally distributed

$$\chi^2 = \sum_{\text{isotherms}} \sum_{\text{points}} (\theta_{obs} - \theta_{calc})^2/\sigma_\theta^2 + \sum_{\substack{\text{calorimetric} \\ \text{runs}}} \sum_{\text{points}} \left[\left(\frac{dq}{d\bar{n}}\right)_{obs} - \left(\frac{dq}{d\bar{n}}\right)_{calc}\right]^2 \Big/ \sigma^2_{dq/d\bar{n}}. \quad (18)$$

The above equation defines the quantity χ^2 which must be minimized in respect to the eight adjustable constants. The first problem is the evaluation of the statistical weights. $\sigma^2_{dq/d\bar{n}}$ is constant within any one data set. Moreover, it changes little regardless of whether a data set is fitted by itself or along with other data. Thus we asssume a plausible set of four binding constants, fit four binding enthalpies to each calorimetric data set alone, and note the standard deviation of the observed data about the best-fitted line. This estimates $\sigma^2_{dq/d\bar{n}}$ for that calorimetric data set. Similarly, we fit four equilibrium constants to each binding curve and evaluate σ^2_θ for those data.

This enables χ^2 to be evaluated and the test of the validity of the assigned weights is that χ^2 approximately equals the degrees of freedom of the system, namely the total number of points in all data sets minus eight, the number of adjustable parameters. For example, with 102 degrees of freedom, we obtained χ^2 ranging from 95 (Adair) to 140 (MWC). These data would suggest that relative to the MWC formalism, the Adair model fits the data significantly better at about the 90% confidence level. On the other hand, any breakdown of symmetry in the MWC model, such as must arise from functional inequivalences of α and β ubunits, will necessitate addition of a fourth adjustable parameter to the MWC formalism. Such a situation does not alter the basic MWC view of the allosterism as resulting from gross ligand affinity differences of conformers in equilibrium. Thus this aspect of our data should not be taken as supporting either model in preference to the other. Once the weights have been evaluated for each data set, χ^2 is then a function of the experimentla data, four heats, and four binding constants referred to 25°C. We desire to minimize χ^2 with respect to these eight parameters subject to two conditions; first, all equilibrium constants are non-negative and, second, any constant K at a temperature different from 25°C is equal to $K_{298}\exp[-(\Delta H/R)(1/T - 1/298)]$. Complete mathematical expansion of χ^2 shows it to be an intractable non-linear function and experience suggests that attempts to linearize such models prove uniformly unsatisfactory. Only when the dependent observables (θ or $dq/d\bar{n}$) are directly fitted in terms of the independent ones (x_t or $\bar{n}$) can the data be processed toward a least-squares fit in a manner both unbiased and stable towards oscillation. This is easily accomplished numerically by application of the algorithm of Marquardt [15], a technique which interpolates between parabolic and gradient searches in parameter space. Moreover, boundary conditions such as the non-negativity of the K may be imposed, normally adjustable parameters may be fixed at some desired value, and error estimates in the fitted constants are produced.

We would like to stress that this overall approach represents, in our opinion, the optimum practical means of extracting free energy and enthalpy changes at once from an ensemble of binding curve and calorimetric experiments having the error characteristics described above.

4. Results and discussion

The CO binding isotherms obtained at 4° and 20° are shown in fig. 1. The smooth curves were calculated from the best-fitted thermodynamic parameters obtained by simultaneous analysis of calorimetric and equilibrium data. These curves are identical regardless of which model, Adair or MWC, is used to fit the data. The slight systematic right shift of the fitted 4° curve relative to the experimental points reflects the influence of calorimetric evidence. This evidence contains the temperature dependence of the isotherms, and, in particular, forces the 4° fitted curve to the right of where the isotherm data alone might suggest.

Fig. 2 illustrates the gas-liquid microcalorimetric measurement of the differential heat of CO binding by the hemoglobin. The smooth curve is cal ulated using both equilibrium and calorimetric data and fits the calorimetric data excellently with a standard deviation of ~0.5 kcal (mol CO)$^{-1}$ for individual differential points. The average heat of ligation is known to much greater precision, namely -2.99 ± 0.07 kcal (mol CO)$^{-1}$. Results obtained in maleate and bis-tris buffers, with heats of protonation of +0.8 and −6.7 kcal (mole H^+)$^{-1}$, respectively, cannot be distinguished in the figure. This indicates directly that there is no net proton release upon ligation. The same conclusion was found by Binotti et al. [14] from the absence of any influence of pH upon the CO binding isotherms.

The detailed thermodynamic functions obtained

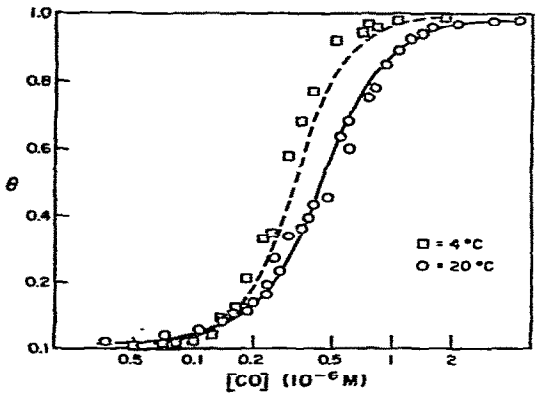

Fig. 1. CO-binding isotherms of Trout Hb I in 0.1 M sodium phosphate buffer, pH 6.8, at 4° and 20°C. The smooth curves result from the overall data fitting procedure.

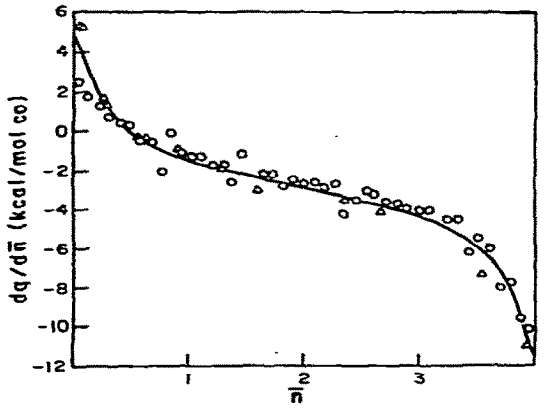

Fig. 2. Differential heat of CO binding to Trout Hb I at 25°C as function of average number $\bar{n}$ of CO molecules bound per tetramer. Circles and triangles denote experiments in 0.05 M bis-tris chloride, pH 7.4, and 0.2 M sodium maleate, pH 7.1, buffers, respectively. The smooth curve results from simultaneous analysis of all data, both isotherm and calorimetric.

by the fitting procedures are indicated in the tables 1, 2, and 3. Table 1 gives the equilibrium constants both for the Adair reaction steps and for the reactions of the MWC model for the three temperatures at which various experiments were performed. The error estimates are given directly by the Marquardt fitting procedure and are comparable in origin to those obtained in linear least-squares procedures [16]. As such, they certainly underestimate the actual errors involved; but, because there are eight adjustable parameters being fitted, the more realistic approach to error estimation, namely application of the F-test [15,16] is out of the question due to the computational time and complexity which such a procedure would entail in this case. The most salient feature of these equilibrium constants is the strong difference in the temperature dependences of the constants, this being a striking indication of the temperature dependent cooperativity which hemoglobin trout I exhibits. For example, the ratio $16K_4/K_1$ or, alternatively, k_R/k_T, varies from 25 at 25° to 200 at 4°. One should note that the Adair and MWC constants presented here result from independent fitting procedures. While the MWC binding polynomial can be expanded (see (1) for equations) to give quantities

Table 1
Stepwise binding constants for reaction of CO with hemoglobin trout I

Equilibrium constant [a]	25°	20°	4°
K_1	$(1.49 \pm 0.17) \times 10^6$ M^{-1}	$(1.29 \pm 0.15) \times 10^6$ M^{-1}	$(0.80 \pm 0.09) \times 10^6$ M^{-1}
K_2	$(1.23 \pm 0.19) \times 10^6$ M^{-1}	$(1.22 \pm 0.19) \times 10^6$ M^{-1}	$(1.21 \pm 0.19) \times 10^6$ M^{-1}
K_3	$(3.82 \pm 0.59) \times 10^6$ M^{-1}	$(4.49 \pm 0.69) \times 10^6$ M^{-1}	$(7.80 \pm 1.20) \times 10^6$ M^{-1}
K_4	$(2.85 \pm 0.24) \times 10^6$ M^{-1}	$(3.96 \pm 0.33) \times 10^6$ M^{-1}	$(12.21 \pm 1.02) \times 10^6$ M^{-1}
L	$(7.13 \pm 2.23) \times 10^{-4}$	$(3.05 \pm 0.95) \times 10^{-4}$	$(0.16 \pm 0.05) \times 10^{-4}$
k_T	$(0.43 \pm 0.04) \times 10^6$ M^{-1}	$(0.39 \pm 0.04) \times 10^6$ M^{-1}	$(0.27 \pm 0.03) \times 10^6$ M^{-1}
k_R	$(12.99 \pm 0.96) \times 10^6$ M^{-1}	$(17.54 \pm 1.29) \times 10^6$ M^{-1}	$(49.31 \pm 3.64) \times 10^6$ M^{-1}

[a] The Adair K_i cited here are the equilibrium constants for the reactions $Hb(CO)_{i-1}(aq) + CO(aq) \rightleftharpoons Hb(CO)_i(aq)$. The MWC reaction parameters, k_T and k_R, are the *intrinsic* binding constants for CO to T- and R-conformation hemoglobin sites, respectively, while L is the equilibrium constant for the $T_0 \rightleftharpoons R_0$ conformational change.

Table 2
Adair stepwise thermodynamic parameters for reaction of hemoglobin trout I with CO at 25°C

Adair	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	$T\Delta S^\circ$ (kcal mol^{-1})	ΔS° (cal deg^{-1} mol^{-1})
1 [a]	−8.42 ± 0.07	4.85 ± 0.35	13.27 ± 0.36	44.5 ± 1.2
2	−8.30 ± 0.09	0.10 ± 0.74	8.40 ± 0.75	28.2 ± 2.5
3	−8.97 ± 0.09	−5.57 ± 0.76	3.40 ± 0.77	11.4 ± 2.6
4	−8.80 ± 0.05	−11.36 ± 0.39	−2.56 ± 0.39	−8.6 ± 1.3
1 [a,b]	−7.60 ± 0.07	4.85 ± 0.35	12.35 ± 0.36	41.8 ± 1.2
2	−8.06 ± 0.09	0.10 ± 0.74	8.16 ± 0.75	27.4 ± 2.5
3	−9.21 ± 0.09	−5.57 ± 0.76	3.64 ± 0.77	12.2 ± 2.6
4	−9.62 ± 0.05	−11.36 ± 0.39	−1.74 ± 0.39	−5.8 ± 1.3

[a] The Adair reaction i cited here is defined as $Hb(CO)_{i-1}(aq) + CO(aq) \rightleftharpoons Hb(CO)_i(aq)$.
[b] In these reactions the statistical entropic term equal to $-R \ln((5-i)/i)$ has been subtracted from all appropriate quantities.

formally identical to the Adair constants, the values obtained by such calculation do not precisely agree with the Adair constants here presented, which result from independent analysis.

Table 2 presents the Adair stepwise thermodynamic parameters for the binding of CO by the hemoglobin at 25°C. The upper section of the table represents the true Adair steps while the lower portion includes corrections for the statistical entropic terms. Thus the free energy changes in this part of the table relate to the "intrinsic" binding constants and are directly comparable with those for k_T and k_R. Several points are apparent from this table. We note first the large compensation effect between the widely varying enthalpic and entropic terms to yield approximate constant stepwise free energy changes. For example, the enthalpy changes for binding the first and fourth ligands are +4.9 and −11.4 kcal (mol CO)$^{-1}$, respectively, yet the intrinsic free energy changes are much less dissimilar at −7.6 and −9.6 kcal (mol CO)$^{-1}$, respectively. A second point concerns the substantial increase in ligand binding free energy which occurs between the second and third reaction steps. This reflects the crossover point of 2.1 in the MWC model at which the protein shifts from the T to the R manifold. Finally, enthalpic factors are seen to govern the overall thermodynamics of ligand binding, since the variation of enthalpy changes is very nearly linear from one reaction step to the next and in the same direction as the free energy changes. In a gross sense, all the thermodynamic parameters vary more or less linearly from step to step and in directions determined by enthalpy changes and opposed in part by entropic compensation. We should underscore here that the temperature dependence in CO-binding cooperativity which hemoglobin trout I exhibits is due to the great differences in ligand binding enthalpies for the various Adair steps.

Table 3 presents the MWC thermodynamic parameters for CO-binding to the hemoglobin. As Wyman et al. [1] pointed out must be the case, the thermodynamic functions for T- and R-state ligation very

Table 3
Monod-Wyman-Changeux thermodynamic parameters for reaction of hemoglobin trout I with CO at 25°C

Reaction	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	$T\Delta S^\circ$ (kcal mol^{-1})	ΔS° (cal deg^{-1} mol^{-1})
$T_0 \rightleftharpoons R_0$	4.29 ± 0.18	29.44 ± 2.15	25.15 ± 2.16	84.4 ± 7.2
T-ligation [a]	−7.68 ± 0.06	3.67 ± 0.39	11.35 ± 0.39	38.1 ± 1.3
R-ligation [a]	−9.70 ± 0.04	−10.42 ± 0.54	−0.72 ± 0.54	−2.4 ± 1.8

[a] The thermodynamic parameters in these rows refer to the reaction of a molecule of CO with a single arbitrary site on a T- or R-conformation hemoglobin molecule.

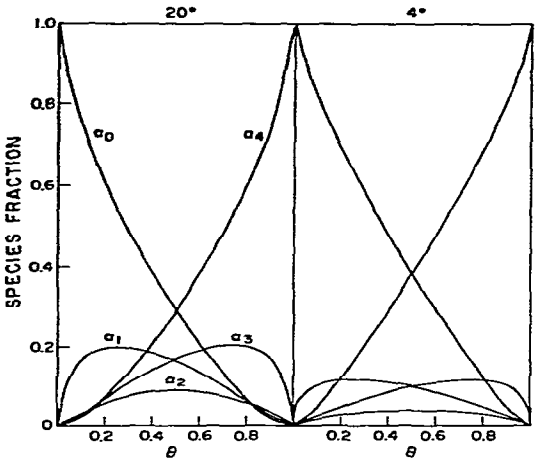

Fig. 3. Fractions α_i of trout Hb I molecules binding to 4 molecules CO (according to the Adair formalism) as function of extent θ of reaction at 20° and 4°C. The maximum value of the species fraction α_i occurs as $\theta = i/4$.

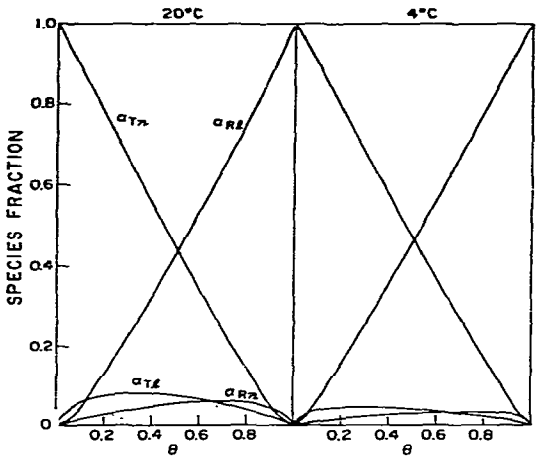

Fig. 4. Fractions α of liganded and non-liganded Hb trout I sites on molecules in the T- and R-conformations. Liganded and non-liganded species are denoted by subscripts ℓ and n, respectively, while subscripts T and R denote protein conformation.

nearly equal those for the first and fourth Adair steps, respectively. The $T_0 \rightarrow R_0$ conformational transition is strongly endothermic (29.4 kcal mol^{-1}), which contributes to the increased cooperativity at low temperatures. This enthalpy change is considerably larger than that for human hemoglobin A at pH 7.4 of 10.8 kcal (mol Hb)$^{-1}$ found using van 't Hoff techniques by Imai and Tyuma [17] and of 9 kcal (mol Hb)$^{-1}$ estimated by comparative calorimetric studies [9]. A major consistency in this and previous studies is the large positive entropy change for the T → R transition at pH 7.4 which probably reflects increased exposure to solvent of protein hydrophobic residues in the more open or "relaxed" R-conformation.

In fig. 3 we show the fractions of Adair species containing various numbers of ligands as a function of degree of reaction at two temperatures. If the system had no cooperativity then the maxima for the species fractions α_1, α_2, and α_3 would be at 0.422, 0.375, and 0.422, respectively. Increasing cooperativity is manifested by the decrease in these maxima as one passes from 20° to 4°. Complete cooperativity would result in the limit of no intermediate species fractions. The MWC model presents a quite different picture. This is illustrated in fig. 4.

In this representation the species are classed as ligated or unligated T or R forms. The fractions of these four groups are shown as the extent of reaction proceeds at two different temperatures. The origin of the cooperativity lies in a series of events where first the unligated state is dominantly T, and as ligation proceeds, the R-ligated and T-unligated forms slowly increase, each going through a low maximum value. In the completely non-cooperative situation α_{Tn} and α_{Rn} would be straight lines decreasing to zero as the reaction runs to completion, while $\alpha_{T\ell}$ and $\alpha_{R\ell}$ would increase as straight lines from initial values of zero.

The differential heat of ligand binding, that is, the heat of per mole of CO ligation, can be expressed in terms of the contributions of the various component reactions. In the case of the Adair representation the reaction heats are for the stepwise processes and the results are depicted in fig. 5. Tne differential functions $df_i/d\bar{n}$ (fig. 5 bottom) which govern the contributions of the reactions are highly symmetric and all steps are significant. The multiplication of the differential functions by their appropriate enthalpies of reaction is shown in the top of fig. 5. The cumulative effects of these contributions is the calculated differential heat of ligand binding shown in fig. 2. It is worth noting that any stepwise property change, such as

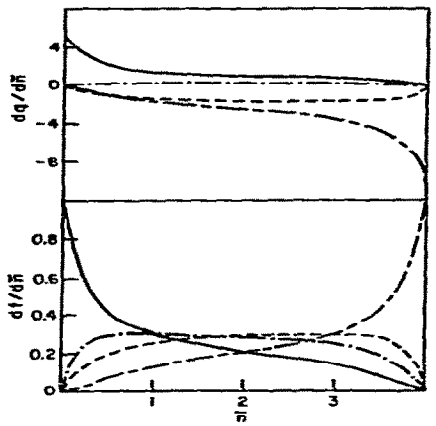

Fig. 5. (Lower): Adair functions $df_i/d\bar{n}$ which determine shape of differential heat of ligation versus $\bar{n}$ curve. The contributions due to first through fourth ligation steps are indicated, respectively, by ——, –.–., - - -, and ——. (Upper): Products of the Adair functions $df_i/d\bar{n}$ times the stepwise heats of CO binding ΔH_i. Each curve represents the contribution of the enthalpy change of a single Adair step to the observed calorimetric data, which would be represented by the sum of the four curves presented here (see smooth curve in fig. 2). The various Adair steps are represented by the same line symbols as in the lower graph.

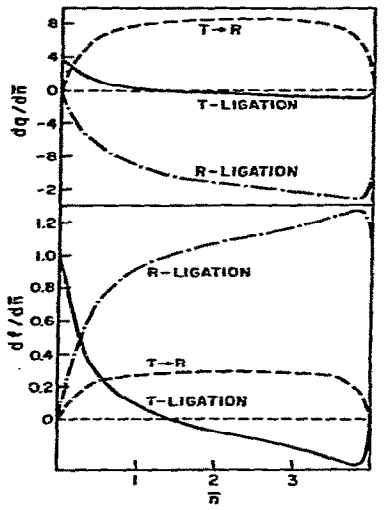

Fig. 6. (Lower): Functions representing contribution *per unit heat of reaction* of the three MWC reaction processes to the differential heat of CO binding as a function of average number $\bar{n}$ of ligands bound. (Upper): Products of the above functions times the heat changes of the respective three MWC reaction processes (see table 3). Each curve represents the contribution of the enthalpy change for a single reaction process to the observed calorimetric data. The predicted calorimetric results would thus be represented as the sum of the three curves presented here (see smooth curve of fig. 2).

moles of proton release per moles of ligand bound, etc., could have been multiplied times the differential functions to obtain each step's contribution to the total differential change in that property with extent of reaction.

The similar calculations based on the MWC model are shown in fig. 6 (bottom). The term corresponding to T-state ligation decreases from an initial value of 1 through 0 and ultimately becomes negative as ligated T-conformation sites undergo conformational change to R. The integral of this function over the reaction coordinate is zero. The function representing R ligation is symmetric with respect to the function previously described since the sum is unity. It starts from zero and increases to a range of values greater than one in which range ligand-induced conformational changes yield more ligated R conformation sites than the total moles of ligand added. This function integrates to four. The contribution from the conformational transition is zero at both ends of the reaction and, being tightly linked to ligand binding, is broadly flat over the majority of the reaction. This function integrates to unity which reflects a nearly complete conformational transition of the tetramer from T to R.

In fig. 7 we have depicted the overall thermodynamic results for the MWC model for hemoglobin trout I. Satisfactory agreement is found with the overall values obtained earlier [1] by a less rigorous analysis of the experimental data. The various states of ligation are represented by the free energy manifold of the T and R forms at 25°C, the T_0 state having been assigned zero free energy. Since the T_2 and R_2 levels are nearly equal in energy, it is at this extent of reaction that the major switch in allosteric forms occurs. On the right is depicted the standard state free energy and enthalpy changes for ligation and conformational transition of the R and T forms.

It has frequently been pointed out (see for example Shulman et al. [8] that the MWC and

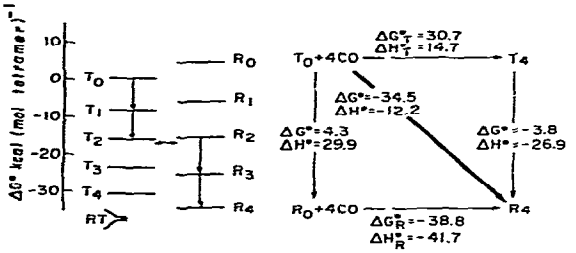

Fig. 7. (Left): Free energy manifolds for CO binding by hemoglobin trout I according to the MWC model. The manifolds are equal in energy at $i \approx 2.1$ and are symmetrically disposed one to another. (Right): Thermodynamic cycle for CO binding and associated allosteric transitions by hemoglobin trout I at 25°C. For visual clarity, entropy changes are omitted. The bold faced arrow indicates the main course of the ligation reaction as experimentally observed.

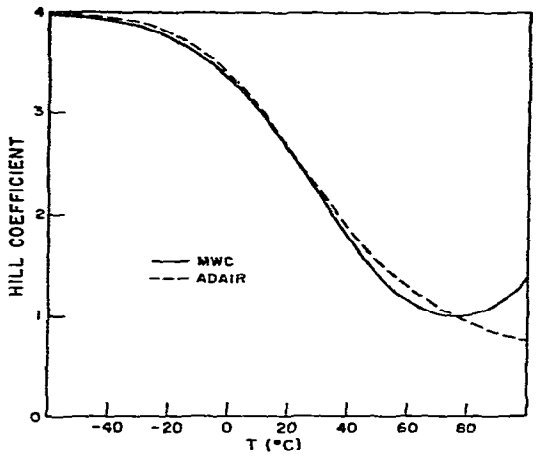

Fig. 8. Temperature dependence of the Hill coefficient for CO binding of hemoglobin trout I. The solid line is that predicted by the MWC model while the broken line is the prediction of the Adair stepwise reaction scheme. In both cases the thermodynamic parameters used are those determined from the experimental data and listed in tables 2 and 3.

Adair models for hemoglobin cooperativity are conceptually very different and have quite distinct implications about, for example, the types of partially-ligated intermediates present. Generally speaking, however, the differences in the models do not predict substantial differences in readily-observable system properties. The interesting system of trout hemoglobin I does contain a striking prediction of how stepwise and concerted models might in theory at least be clearly differentiated by simple experiment. Wyman has pointed out [19] that allosteric models for homotropic systems can yield only positive cooperativity. Stepwise schemes, on the other hand, are not so constrained and negatively-cooperative systems thus describable are well-known, as for example, the protonation of polybasic anions. The Hill coefficient [16] defined as $(\partial \ln(\theta/(1-\theta)))/\partial \ln x)$ provides a useful, time-honored measure of overall ligand binding cooperativity in tetrameric hemoglobins, values between one and four implying positive cooperativity, a value of unity implying a non-cooperative system, and values less than one implying negative cooperativity.

The thermodynamic parameters shown in table 2 for the Adair stepwise scheme clearly indicate that, at temperatures greater than about 75°C, the intrinsic CObinding constants of the reaction will *decrease* with each successive reaction step. The Hill coefficient will, in this situation, decrease monotonically from highly-cooperative values (≈3) near room temperature through unity at ≈75°C to values less than unity at still higher temperatures. In the MWC model, on the other hand, the thermodynamic parameters of table 3 indicate that at a temperature of ≈75°, the T and R manifolds (see fig. 7) will be essentially indistinguishable, both in spacing and in relative energy. This situation implies a non-cooperative system with a Hill coefficient of one. On the other hand, at still higher temperatures, the T_0 level will be higher in energy than R_0 and, moreover, the spacing of levels in the T-manifold will be greater than that in the R-manifold. This describes a *cooperative* allosteric system precisely analogous to that at room temperature, save that the two conformational states have become reversed in affinity and in the equilibrium constant between their deoxy forms. Thus, the Hill coefficient is predicted in this trout hemoglobin system to rise to values greater than one at temperatures above 75°C. Fig. 8 shows the values of the Hill coefficient which the two models predict, assuming measurements could be made from −50°C to +100°C. While no substantive differences in this measure of cooperativity would be expected to be discernable below, say, 40°C, at higher temperatures the stepwise Adair model would predict a monotonic decrease in

the Hill coefficient while the MWC model predicts a minimum value followed by a monotonic increase in the parameter at still higher temperatures. Since measurements in this temperature range are clearly unrealistic, the hemoglobin trout I system does not afford an opportunity to differentiate experimentally the two models by this technique. On the other hand, there may well be hemoglobin systems which exhibit low apparent cooperativity in the physiological temperature range and which might possess stepwise enthalpy changes comparable to trout. If such a system existed one could in effect examine the right hand portion of fig. 8 at experimentally attainable temperatures. This would permit, at least in this special case, a clear test of the allosteric model as opposed to a stepwise scheme.

Brunori [21] has discussed the physiological roles of the various trout hemoglobins and has noted the relative temperature independence of the hemoglobin trout I oxygen dissociation curve. He observes that this affords a system capable of supplying oxygen to the tissues independent of the external temperature. These data here presented show that the adaptation of the trout, as a poikilothermic organism, to a varying-temperature environment is even more sophisticated. The substantial increase in ligand binding cooperativity which hemoglobin trout I exhibits at low temperatures must enhance the efficiency of oxygen unloading and hence, of oxygen transport at these lower temperatures. This increased efficiency of oxygen transport must offset the general kinetic slowdown which the trout's oxygen transport system encounters at low environmental temperatures.

We should, in conclusion, stress the general applicability of the methodology presented here to the analysis of data on other processes linked to ligand binding. For example, the number of protons released in the various component reactions of hemoglobin ligation could be evaluated by simultaneous analysis of two types of data sets. First, proton release or uptake could be studied at constant pH as a function of extent of reaction; this would be analogous to the calorimetric data here presented. Simultaneously, ligand binding isotherms at various pH values would, like the isotherm measurements, at various temperatures, provide the complementary data for optimal determination of stepwise proton changes.

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