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Discontinuous Release of Heat at Successive Steps of Oxygenation in Human and Bovine Hemoglobin at pH 9.0†

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ABSTRACT: We have measured the temperature dependence of the oxygen-binding isotherms of human and bovine hemoglobin at pH 9.0 in 0.1 M borate buffer. In both hemoglobins the ionization of the Bohr protons is finished at this pH; therefore, their heat does not interfere with the measurements. Two sets of curves have been obtained, which have been analyzed by either singular or global procedures for estimating the enthalpy changes of subsequent steps of oxygenation. The data indicate that in human hemoglobin the reaction with oxygen is enthalpy driven for steps 1, 2, and 4 while it is entropy driven for step 3. In bovine hemoglobin this phenomenon is even more evident: steps 2 and 4 are enthalpy driven while steps 1 and 3 are entropy driven. The discontinuous distribution of heat at subsequent steps of oxygenation suggests that the T to R transition in hemoglobin is not a monotonic process and involves conformations with novel characteristics.

Cooperativity of oxygen binding in hemoglobin implies the transition from a low to a high-affinity conditions, which indicates a progressive modulation of the free energy of the reaction. The intrinsic reactivity of the heme iron for oxygen is constant; therefore, the changes are due to allosteric modulations of the thermodynamic parameters of the system. Thus, measuring the temperature dependence of the oxygen affinity may provide information on the heat of the conformational changes that modulate the system. The distribution of these heats at subsequent steps of oxygen binding may reveal critical conformational events of the system during oxygenation.

The data available in the literature are uncertain whether the enthalpy of oxygenation is linear with successive oxygenation steps. Data presented by Imai (1979) indicate that at alkaline pH the enthalpy is sharply less exothermic for the third step of oxygenation. Calorimetric measurements of Parodi-Monreale et al. (1987) indicate linearity for steps 1,

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2, and 4 while remaining uncertain for step 3, which is very difficult to detect in their measurements. Atha and Ackers (1974) report calorimetric data that could equally well be explained by a linear or nonlinear release of heat with oxygen binding.

In view of the relevance of this problem with regard to the sequence of conformational events during oxygenation, we attempted to clarify the situation by measuring the temperature dependence of oxygenation of two different hemoglobin systems. We explored and compared the behavior of human and bovine hemoglobins, which have a different allosteric sensitivity to effectors (Fronticelli et al. 1988; Razynska et al., 1988), so as to have two different and totally independent sets of experiments on which to base the interpretation of the data. The experiments were conducted at alkaline pH so as to avoid the heat of ionization of the Bohr protons, which would complicate the analyses and interpretation of the data. Also, very conveniently, at alkaline pH, the overall constant β_3 was measurable, at variance with neutral pH, where it acquires uncertain values very near 0.0 (Gill et al., 1987; Vandegriff et al., 1989). This allowed meaningful numerical analyses of the data.

In both systems the data indicate that the release of heat at the subsequent steps of oxygen binding is discontinuous.

Materials and Methods

Human oxyhemoglobin was prepared from washed red cells and purified by HPLC chromatography as previously described (Bucci et al., 1988). It was stored at −80 °C in water after being recycled through mixed bed resin cartridges so as to eliminate all organic and inorganic ions.

Purified bovine oxyhemoglobin was obtained from Biopure (Boston, MA). This protein was found to be chromatographically and electrophoretically pure and therefore was used without further purifications. It was stored at −80 °C in water after being recycled through a mixed bed resin cartridge.

Oxygen equilibria were measured with the dilution method of Dolman and Gill (1978), with protein concentrations between 30 and 60 mg/mL. The temperature of the experiments was controlled to an accuracy of 0.05 °C with a Lauda bath RMS. The changes in optical denisty were monitored with accuracy to 10^{-5} OD units with an AVIV 14DS spectrophotometer at the following wavelengths: 414, 438, 441, or 577 nm. Reducing enzymes were not used in the samples because the absorption spectra of the samples before and after the experiment were superimposable. Control experiments performed in the presence of reducing enzymes (Hayashi et al., 1973) gave results very similar to those obtained in the absence of enzymes. The initial content of ferric hemoglobin was below 3%.

The buffer used was 0.1 M borate buffer at pH 9.0. Measurement of pH was performed on solutions brought to the desired temperature with a tolerance of ±0.05 pH unit. The pH of borate buffer was insensitive to temperature.

Treatment of the Data

For each individual measurement changes in optical absorbance upon stepwise changes of P_{O_2}[1] fit to the equation

$$\Delta OD_i = \Delta OD_T \, \Delta\theta_i \qquad (1)$$

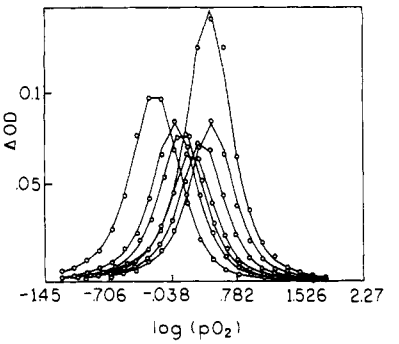

FIGURE 1: Global analysis of the temperature-dependent isotherms of human hemoglobin at 20.2, 25.6, 27.6, 30.4, 33.4, 35.4, and 36.4 °C, respectively, in 0.1 M borate buffer at pH 9.0. Protein concentration was near 50 mg/mL.

where ΔOD_i is the absorbance change at each dilution step, $\Delta\theta_i$ is the corresponding change in fractional saturation of hemoglobin with oxygen, and ΔOD_T is the total absorbance change obtained in going from deoxy- to oxyhemoglobin. The fractional saturation θ was computed by (Wyman, 1964)

$$\theta = \mathrm{d} \ln P / 4\mathrm{d} \ln X \qquad (2)$$

P is the binding polynomial

$$P = 1 + \beta_1 X + \beta_2 X^2 + \beta_3 X^3 + \beta_4 X^4 \qquad (3)$$

where X is the free ligand concentration (i.e., P_{O_2} (mmHg)) and β_i are the overall affinity constants defined as

$$K_i = i\beta_i/(5 - i)\beta_{i-1} \qquad i = 1\text{–}4 \qquad (4)$$

where K_i are the intrinsic individual affinity constants at each step of the titration with oxygen.

Analysis of the isotherms gave the four overall constants β_i and the median ligand activity $P_m = (\beta_4)^{-0.25}$ at each temperature. For estimation of the overall constants $\beta_{25,i}$ and their enthalpies $\beta H_{25,i}$ at the reference temperature (25 °C), the isotherms obtained at the various temperatures were simultaneously fit to the binding polynomial P_h

$$P_h = 1 + \sum_i \beta_{25,i} X^i \exp[(\beta H_{25,i}/R)(1/T_e - 1/298.2)] \qquad i = 1\text{–}4 \qquad (5)$$

where $\beta_{25,i}$ are the overall binding constants at the reference temperature (25 °C), $\beta H_{25,i}$ are the enthalpies of the overall constants, T_e is the temperature of the various experiments in Kelvin, 298.2 is the reference temperature in Kelvin, and R is the gas constant. The enthalpies $\Delta H_{25,i}$ of the intrinsic constants at the reference temperature of 25 °C were computed from the overall enthalpies by

$$\beta H_{25,i} = \sum_i \Delta H_{25,i} \qquad i = 1\text{–}4 \qquad (6)$$

Computer analyses were performed according to a nonlinear least-square procedure described by Gill et al. (1987).[2]

Results

Oxygen-binding isotherms for human and bovine hemoglobin were measured at seven and five different temperatures, respectively. Computer analyses were performed on a local mode for each single curve and on a global mode for each set of curves. The sets of curves used for global analyses are shown

[1] Abbreviations and glossary: P_m, median activity of oxygen; P_{O_2}, partial pressure of hemoglobin; local analysis and parameters, individual analysis and resulting parameters of single oxygen-binding isotherms; global analysis and parameters, simultaneous analysis and resulting parameters of a set of oxygen-binding isotherms with common parameters.

[2] Only raw data were used for the numerical analyses. It is our firm opinion that weighing of experimental data performed either following a predetermined scheme or by logarithmic transformation or any other numerical massage only converts objective data into controversial opinions.

Table I: Local (L) and Global (G) Overall Adair Constants Describing the Oxygen-Binding Isotherm of Human Hemoglobin in 0.1 M Borate Buffer at pH 9.0[a]

	20.2 °C		25.6 °C		27.6 °C		30.4 °C		33.4 °C		35.4 °C		36.4 °C		25 °C
	L	G	L	G	L	G	L	G	L	G	L	G	L	G	G
β_1	1.80	1.33	1.03	0.820	0.830	0.700	0.620	0.540	0.520	0.420	0.299	0.350	0.371	0.330	0.859
sd	10	<1	13	<1	8	<1	13	<1	8	<1	29	<1	15	<1	1
β_2	1.63	1.71	0.312	0.400	0.190	0.250	0.107	0.117	0.065	0.055	0.039	0.034	0.024	0.026	0.469
sd	11	<1	21	<1	14	<1	25	<1	14	<1	40	<1	41	<1	1
β_3	0.915	0.842	0.243	0.180	0.170	0.110	0.052	0.049	0.021	0.022	0.010	0.013	0.013	0.010	0.214
sd	23	<1	18	<1	8	<1	19	<1	13	<1	36	<1	18	<1	1
β_4	2.00	2.13	0.204	0.207	0.095	0.097	0.033	0.028	0.011	0.008	0.003	0.003	0.003	0.002	0.263
sd	6	<1	8	<1	4	<1	7	<1	5	<1	14	<1	9	<1	1

[a] The overall constants β_i are in $Torr^{-i}$. The data at the reference temperature (25 °C) are from global analysis only. The standard deviations (sd) are given in percent of the estimated value (±%).

Table II: Local (L) and Global (G) Estimated Overall Adair Constants Describing the Oxygen-Binding Isotherm of Bovine Hemoglobin in 0.1 M Borate Buffer at pH 9.0[a]

	20.0 °C		25.5 °C		28.45 °C		30.5 °C		36.5 °C		
	L	G	L	G	L	G	L	G	L	G	G
β_1	0.500	0.360	0.460	0.340	0.310	0.330	0.290	0.320	0.310	0.300	0.344
sd	4	<1	11	<1	12	<1	15	<1	19	<1	>1
β_2	0.108	0.094	0.020	0.026	0.014	0.013	0.003	0.008	0.002	0.002	0.029
sd	1	<1	24	<1	46	<1	82	<1	30	<1	>1
β_3	0.003	0.008	0.003	0.004	0.003	0.003	0.002	0.002	0.0004	0.001	0.005
sd	170	<1	120	<1	43	<1	30	<1	75	<1	>1
β_4	0.024	0.019	0.005	0.004	0.002	0.002	0.001	0.001	0.0003	0.0003	0.005
sd	1	<1	6	<1	6	<1	8	<1	9	<1	1

[a] the overall constants β_i are in $torr^{-i}$. The data at the reference temperature (25 °C) were obtained from global analysis only. The standard deviations (sd) are given in percent of the estimated value (±%).

Table III: Thermodynamic Parameters Associated with the Intrinsic Affinity Constants at Each Step of Oxygenation of Human Hemoglobin, in 0.1 M Borate Buffer at pH 9.0, at 25 °C

	step 1	step 2	step 3	step 4
K (mM^{-1})	139.4 ± 1.0[b]	235.9 ± 1.0	445.5 ± 1.1	3193.2 ± 1.0
ΔH^a (kcal/mol)	−12.6 ± 3.0	−27.7 ± 3.0	0.5 ± 190.0	−22.9 ± 3.0
ΔG (kcal/mol)	−6.9 ± 0.2	−7.2 ± 0.2	−7.6 ± 0.5	−8.8 ± 0.5
ΔS (cal mol^{-1} deg^{-1})	−18.9 ± 0.7	−68.5 ± 4.0	27.2 ± 5.0	−47.3 ± 3.0
$T\Delta S$ (kcal/mol)	−5.6 ± 0.7	−20.4 ± 4.0	8.1 ± 5.0	−14.1 ± 3.0

[a] The average enthalpy value for the four oxygenation steps is ΔH_{av} = −15.7 kcal/mol. [b] The standard deviations are given in percent of the estimated value (±%).

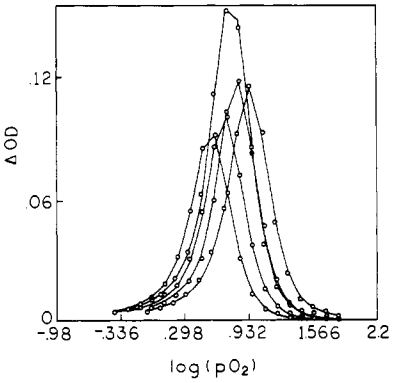

FIGURE 2: Global analysis of the temperature-dependent isotherms of bovine hemoglobin at 20.0, 25.5, 28.4, 30.5, and 36.5 °C, respectively, in 0.1 M borate buffer at pH 9.0. Protein concentration was near 50 mg/mL.

in Figures 1 and 2 for human and bovine hemoglobin, respectively. Tables I and II compare the values of the overall β_i constants estimated by local and global simulations. Clearly, the global mode produced better defined parameters and reduced their standard deviations.

Table IV: Thermodynamic Parameters Associated with the Intrinsic Affinity Constants at Each Step of Oxygenation of Bovine Hemoglobin, in 0.1 M Borate Buffer at pH 9.0, at 25 °C

	step 1	step 2	step 3	step 4
K (mM^{-1})	55.7 ± 1.1	36.3 ± 1.02	159.0 ± 1.1	985.2 ± 2.0
ΔH^a (kcal/mol)	0.8 ± 3.0	−35.8 ± 5.0	23.0 ± 3.0	−20.5 ± 2.0
ΔG (kcal/mol)	−6.4 ± 0.2	−6.2 ± 0.5	−7.1 ± 0.5	−8.0 ± 0.5
ΔS (cal mol^{-1} deg^{-1})	24.3 ± 2.0	−99.4 ± 5.0	101.1 ± 8.0	−39.2 ± 6.0
$T\Delta S$ (kcal/mol)	7.2 ± 2.0	−29.6 ± 5.0	30.1 ± 9.0	−11.7 ± 6.0

[a] The average enthalpy value for the four oxygenation steps is ΔH_{av} = −8.1 kcal/mol. [b] The standard deviations are given in percent of the estimated value (±%).

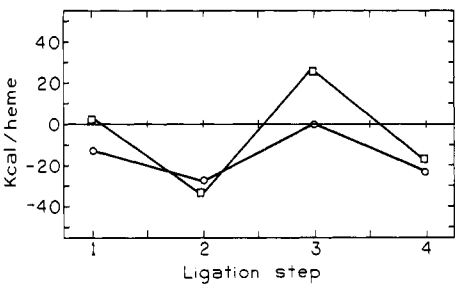

FIGURE 3: Enthalpy change values at subsequent ligation steps of human (O) and bovine (□) hemoglobin.

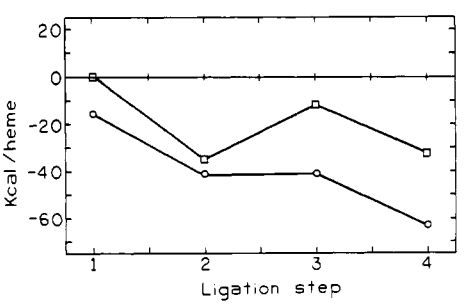

FIGURE 4: Total release of heat at successive oxygenation steps of human (O) and bovine (□) hemoglobin.

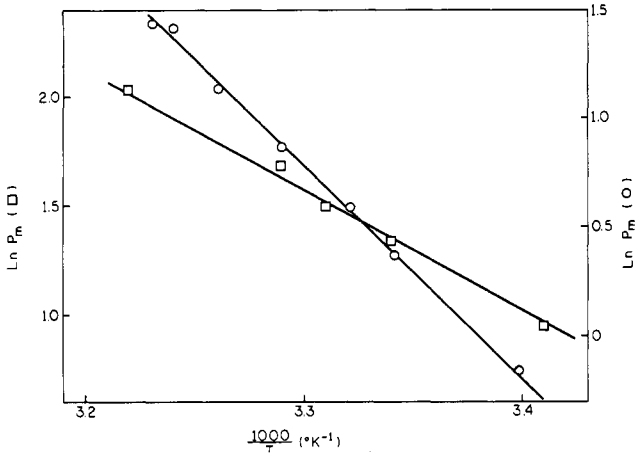

FIGURE 5: van't Hoff plots of the temperature dependence of P_m of human (○) and bovine (□) hemoglobins in 0.1 M borate buffer at pH 9.0. The interpolating lines represent values obtained from global analyses. The left and right ordinate scales are for bovine and human hemoglobin, respectively.

Tables III and IV list the thermodynamic parameters associated with oxygen binding to human and bovine hemoglobin, respectively. The energetics of the ligation process are graphically reported in Figures 3 and 4. They show the value of the enthalpy at each step of oxygenation and the progressive release of heat at subsequent levels of ligation. They clearly show the presence of a much less exothermic reaction for the triligated species of both hemoglobins. This decrease is more pronounced in bovine hemoglobin where, as shown in Figure 3, binding of the third oxygen molecule is even endothermic. Notably, in bovine hemoglobin binding of the first oxygen has an enthalpy close to 0.0. As shown in Tables III and IV in both hemoglobins the free energy change of oxygen binding remains rather constant for the first three steps of oxygenation.

Figure 5 shows the van't Hoff plots of the P_m values computed from local analyses for human and bovine hemoglobin. The data points from local analyses are interpolated by the lines obtained from global analyses. As shown by the Gibbs–Helmholtz equation

$$\mathrm{d} \ln P_m/\mathrm{d}(1/T) = \Delta H^\circ/R \qquad (7)$$

the slopes of the lines correspond to the overall enthalpy of the reaction

$$\mathrm{Hb} + 4\mathrm{O_2} = \mathrm{Hb(O_2)_4} \qquad (8)$$

and are identical with the average enthalpy values shown in the footnotes of Tables III and IV, respectively. These values are consistent with those obtained at pH 7.4, as previously reported (Razynska et al., 1988), and confirm that the overall enthalpy of bovine hemoglobin is significantly less exothermic than that of human hemoglobin.

Discussion

Reports from many laboratories including ours (Razynska et al., 1988; Gill et al., 1987; Vandegriff et al., 1989) show that by using the thin-layer dilution method at neutral pH the overall constant β_3 has a very low uncertain value, near 0.0. Instead, in borate buffer at pH 9.0 the constant β_3 is detectable and statistically meaningful as proven by a standard deviation less than 1% of its value. The larger value of β_3 at alkaline pH indicates the stabilization of the triligated species, which becomes measurable; this phenomenon is associated with a decreased cooperativity, as shown by the value of the Hill's parameter n, which at alkaline pH had a maximum value of 2.3 while it was 2.7 at neutral pH. The reason for the stabilization of the triligated species is not clear at present;

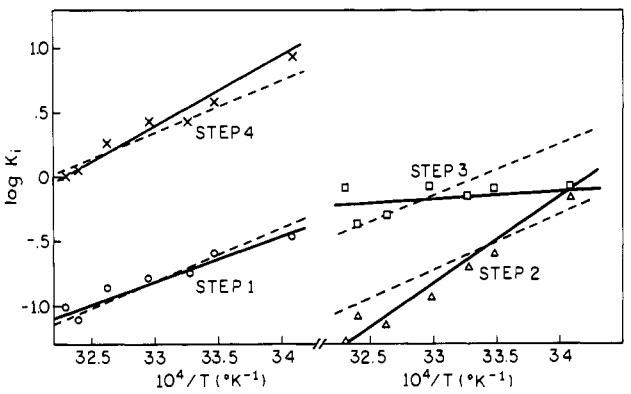

FIGURE 6: Human hemoglobin: comparison of the van't Hoff plots of the intrinsic affinity constants obtained from the local parameters of Table I with those obtained from global analyses. The data points refer to the constants from local analyses; the lines are obtained from global analyses. Dashed lines: data from global analyses with fixed identical enthalpies at each oxygenation step. Continuous lines: data from the global analyses shown in Table I.

nevertheless, it conveniently allowed the estimation of the thermodynamic parameters associated with the overall Adair's constants in eq 5.[3]

With regard to the accuracy of the data, it should be stressed that we have used highly purified hemoglobins, containing nondetectable amounts of ferric hemes, that the superimposition of the spectra at the beginning and end of the experiments proved negligible or no formation of methemoglobin during the measurements, and that the spectrophotometer used in the experiments was accurate to the fifth decimal position of OD units. As mentioned above, the van't Hoff plots at pH 9.0 shown in Figure 5 were very consistent with those obtained in our laboratory at pH 7.4 (Razynska et al., 1990).

The data have been analyzed by singular and by global analyses; while local analyses produced standard deviations of 10% or more of the estimated parameters, examination of Tables I and II shows that global analyses produced standard deviations below 1% of the estimated parameters. It should be stressed that in both sets of data the standard deviations of all parameters progressively decreased with each subsequent addition of a new isotherm into the global analysis. This implies an excellent consistency of the data obtained at the various temperatures for the two hemoglobins. In fact, if errors were affecting any of the isotherms, subsequent addition of those isotherms into global analysis would have prevented meaningful minimizations and produced an increase in the standard deviations of the common parameters. It should also be stressed that the global analyses included isotherms monitored at different wavelengths. This implies that our data were not affected by a possible nonlinearity of optical density changes with oxygen saturation (Parkhurst et al., 1990; Ownby & Gill, 1990).[4]

[3] Preliminary data in our laboratory suggest that in the presence of Cl^- ions the value of β_3 is depressed.

[4] It may be asked what impact would have the presence of dimers on the enthalpy data here presented. Our data were obtained at heme concentrations between 2 and 4 mM. The global analyses failed to show inconsistencies due to the concentration dependence of the overall Adair constants β_i. From the dimer association constants reported by Mills et al. (1976) it is possible to estimate that under our conditions the dimer concentrations varied from 10^{-6} in oxyhemoglobin to 10^{-9} in deoxyhemoglobin. These are negligible quantities. On the basis of the models of Mills et al. (1976), at the protein concentrations used in our experiments, the errors estimated by Johnson and Lassiter (1990) for the overall constants would be 0.15 kcal or less. Even if those models and corrections are accepted, this is between 1 and 2% of the values reported in Tables I and II.

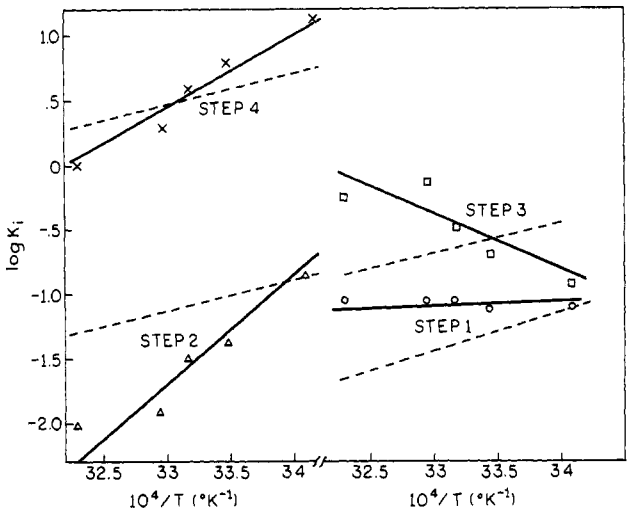

FIGURE 7: Bovine hemoglobin: comparison of the van't Hoff plots of the intrinsic affinity constants obtained from the local parameters of Table II with those obtained from global analyses. The data points refer to the constants from local analyses; the lines are obtained from global analyses. Dashed lines: data from global analyses with fixed identical enthalpies at each oxygenation step. Continuous lines: data from the global analyses shown in Table II.

The question may be posed whether the nonlinearity shown by the data presented here resulted from local minima of the fitting procedures. To explore this possibility, we repeated the global analyses by fixing the enthalpy of each step of oxygenation to the average values listed in the footnotes of Tables III and IV. The standard deviations of the various parameters increased 10–100-fold. Also, Figures 6 and 7 show unequivocally that the data of Tables I–IV do not represent false minima of the global analyses. These figures show the van't Hoff plots of the data points representing the intrinsic constants K_j obtained by using eq 4, from local analysis of individual isotherms interpolated by the lines obtained either from the global analyses listed in Tables I and II (continuous lines) or from the K_j obtained by using fixed enthalpies as described above (dashed lines). The dashed lines did not correspond to the local data, while there was an excellent agreement between the local points and the continuous lines consistent with a nonlinearity of enthalpy release upon oxygenation.

Oxygenation of the heme of hemoglobin is intrinsically an exothermic event driven by the negative enthalpy of the reaction. Therefore, it may be expected that the progressive higher affinity of the reaction, as implied in the cooperativity of oxygen binding, is produced by monotonic increases of the negative enthalpy at each oxygenation step. To the contrary, the data in Figures 3 and 4 show that the release of heat is sharply reversed when the third ligand enters the molecule and the enthalpy acquires a near-zero or a distinct positive value. In bovine hemoglobin the first step of ligation also has a low positive enthalpy.

It appears that the heat exchanges in hemoglobin include endo- and exothermic processes not equally distributed on the subsequent oxygenation steps. In bovine hemoglobin steps 1 and 3 are entropy driven while steps 2 and 4 are enthalpy driven. In human hemoglobin step 3 is entropy driven. This discontinuous distribution of heats must originate from conformational changes that produce exo- and endothermic events superimposed to the intrinsic negative enthalpy of the reaction of the heme iron with oxygen.

Chothia (1974) reports that upon oxygenation additional hydrophobic residues are exposed to the surface of hemoglobin. This is an exothermic event that may explain the enthalpy in excess to the average of steps 2 and 4 in human and bovine hemoglobin.

Oxygen dissociation is an endothermic event. On speculative grounds it can be observed that the triligated species is highly asymmetric in the sense that one unligated subunit is in contact with three ligated ones. This asymmetric situation may justify a negative binding cooperativity consistent with the allosteric model described by Koshland et al. (1966). It can be proposed that the triligated species is so anticooperative that when an oxygen molecule binds to a diligated species it may produce the dissociation of bound oxygen from other hemes of the same molecule. Anticooperativity limited to certain intermediate species would depopulate the intermediates, resulting in positive binding cooperativity of the whole system, as noticed by Plesner (1990) and Di Cera (1990).

At present we can only formulate hazardous speculations for explaining the molecular mechanism of heat release in hemoglobin. The evidence of a discontinuous distribution of heats at subsequent steps of ligation suggests that the transition from the T state to the R state involves the appearance of special conformations for each intermediate species. The alternate occurrence of enthalpy-driven and entropy-driven binding steps implies that these conformations have novel characteristics and do not represent a monotonic transition between the T state and the R state. The drastic reversal of the enthalpy in both human and bovine hemoglobin at the third ligation step may be the main conformational event of the allosteric phenomena associated with ligand binding.

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