# Entropy-Driven Intermediate Steps of Oxygenation May Regulate the Allosteric Behavior of Hemoglobin

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ABSTRACT When the oxygen binding isotherms of human, bovine and fallow deer (Dama-Dama) hemoglobins are measured at different temperatures either by optical or calorimetric techniques, analyses according to the Adair's formalism show that at least one of the intermediate steps of ligation has a positive enthalpy change, i.e., absorbs rather than emitting heat, indicating that it is entropy rather than enthalpy driven (Bucci, E., et al. 1991. *Biochemistry.* 30:3195–3199; Bucci, E., et al. 1993. *Biochemistry.* 32:3519–3526; Johnson, C., et al. 1992. *Biochemistry.* 31:10074–10082; Johnson, C., et al. 1995. *Biophys. Chem.* 59:107–117). This phenomenon is confirmed in systems in which the $\beta$82 lysines of human hemoglobin are covalently cross-linked by acylation with dicarboxylic acids of increasing length, namely the fumaryl (four-carbon-long), adipoyl (six-carbon-long), and sebacoyl (10-carbon-long) residues. Consistently in all of the systems here reported, the enthalpy excursions are masked by compensatory entropy changes, which keep the free energy of ligand binding constant for the first three steps of oxygenation. Furthermore, the cooperativity index and the overall oxygen affinity seem to be correlated to the positive enthalpy excursions of the intermediate steps of ligation. Fumaryl-Hb (hemoglobin cross-linked with a fumaryl residue, four carbons) with the lowest absorption of heat has the highest affinity and lowest cooperativity index. Adipoyl-Hb (hemoglobin cross-linked with an adipoyl residue, six carbons) has the highest absorption of heat and the highest cooperativity index. It appears that nonuniform heat release by the intermediates of oxygenation is part of the allosteric phenomena in hemoglobin systems. There is not enough information that would allow assigning these phenomena to the interplay of the various conformations described for hemoglobin besides the classic T (Fermi et al. 1984. *J. Mol. Biol*. 175:159–174) and R (Shanaan. 1983. *J. Mol. Biol.* 171:31–59), as listed at the end of the Discussion. The possibility cannot be excluded that entropy-driven steps characterize new conformational transitions still to be described.

## INTRODUCTION

After the publication of the fundamental work of Monod, Wyman, and Changeaux (Monod et al., 1965), the thermodynamic concepts of allosteric systems appear so obvious that they are taken for granted. Ligand-linked conformational changes are at the basis of those phenomena, and to a first approximation these systems can be described assuming two conformational states, ligated and unligated. A two-state hypothesis can explain most of the allosteric characteristics of hemoglobin, which is regulated only by equilibrium conditions, dictating when and how ligands can be accepted or released by the protein.

Oxygen transport by hemoglobin in vivo requires a fine-tuned adaptation to physiological needs. This is accommodated by modulations of the positive ligand binding cooperativity and the overall oxygen affinity of the system. The simultaneous optimization of these two parameters requires an unprecedented flexibility of the allosteric parameters, suggesting that only two probable structures are not sufficient to explain the phenomenon. Moreover, two-state concerted models cannot explain the negative binding cooperativity of certain fish hemoglobins (Brunori et al., 1973) and of sebacoyl-Hb (hemoglobin cross-linked with a sebacoyl residue, 10 carbons) (Bucci et al., 1996). Intermediate forms must be hypothesized, as proposed by the model of Koshland (Koshland et al., 1966).

There is evidence that hemoglobin exists in several conformations regulated by the extent of ligation. The classic work of Ackers and colleagues on ferrous-ferric and hybrid intermediates proposes the presence of a third conformation along the pathway of oxygenation of hemoglobin (Ackers and Smith, 1987; Ackers et al., 1992; Huang et al., 1996). A third cooperative state of hemoglobin has been detected by Jayaraman and Spiro (Jayaraman et al., 1995), using ultraviolet Raman spectroscopy. Data obtained by van t'Hoff procedures and by direct calorimetry indicate the presence of enthalpy- and entropy-driven ligation steps in alternating fashion (Bucci et al., 1991, 1993; Johnson et al., 1992, 1995) along the pathway of oxygenation of hemoglobin. This may be consistent with the proposition of additional conformational states of hemoglobin.

To clarify these phenomena, we have conducted a systematic investigation of the thermodynamics of oxygenation of human hemoglobin intramolecularly cross-linked with identically positioned bridges of different lengths. The rationale was to investigate whether internal cross-linking would prevent the formation of intermediate conformational states, so as to reveal their allosteric relevance. The thermodynamic characteristics of the cross-linked hemoglobins, compared to those of natural systems previously investigated by us (Bucci et al., 1991, 1993) and in other labora-

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tories (Johnson et al., 1992, 1995), allow an initial evaluation of the allosteric significance of entropy-driven intermediate steps of oxygenation.

## MATERIALS AND METHODS

Human hemoglobin was prepared from washed red cells and purified as previously described (Bucci et al., 1988). It was stored at −80°C and distributed into vials, which were thawed only once.

Bis-activation of adipic and sebacic acid with 3,5-dibromosalicylate was performed as previously described for polycarboxylic acids (Razynska et al., 1991). Sebacic acid and 3,5-dibromosalicylate were obtained from Aldrich Chemical Co. The purity of the product was determined by elemental analysis (Atlantic Microlab) and by proton NMR spectroscopy. The chemical formulas of the bis(3,5-dibromosalicyl) esters of dicarboxylic acids are shown in Fig. 1.

### Reaction of human hemoglobin with the bis(3,5-dibromosalicyl) esters

To a 6% solution of deoxygenated hemoglobin in 0.05 M borate buffer at pH 9.0, the bis-esters were added in a 2:1 to 4:1 molar ratio to tetrameric hemoglobin. The mixture was kept at 37°C for 90 min with continuous stirring, under a stream of nitrogen. We added sodium dithionite (0.5 mg/ml) to ensure the complete deoxygenation of the solutions. The reaction was stopped by the addition of glycylglycine to a final concentration of 0.1 M, followed by dialysis against 0.1 M glycine at pH 8.0, in the cold for at least 4 h. After this treatment, the incubation mixtures were dialyzed against the buffers necessary for chromatographic purification.

Purification of the cross-linked hemoglobins was performed by anion exchange chromatography on columns packed with DEAE-MCI resin (Mitsubishi), using Waters Delta-Prep 4000 high-performance liquid chromatography equipment. We used gradients formed by 0.015 M Tris-acetate at pH 8.2 and 0.015 M Tris-acetate buffer in 0.2 M Na-acetate at pH 7.7. Repetitive chromatographies were used until electrophoresis on Paragon gels (Beckman) showed the presence of a single component, and reversed-phase chromatography (described below) showed the presence of only two protein components, namely the non-cross-linked $\alpha$-subunits and the cross-linked $\beta$-subunits.

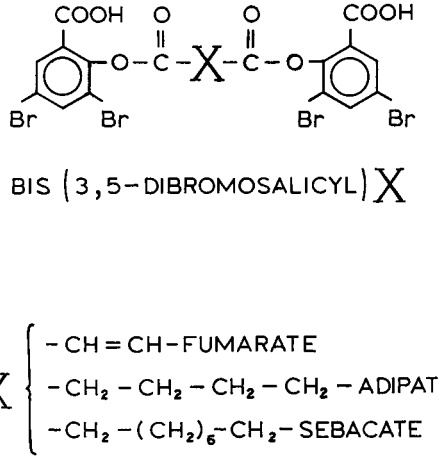


FIGURE 1 Chemical formulas of the cross-linkers used for the chemical treatments of hemoglobin.

Separation of the $\alpha$- and $\beta$-chains and peptide mapping were performed by reversed-phase chromatography, using a C4 Vydac column and a gradient of $CH_3CN$ in 0.1 M TFA. After separation, the $\beta$-chains were pyridylethylated by classic procedures, equilibrated with 0.1 M bicarbonate, and digested with 1-chloro-3-tosylamido-7-amino-2-heptanone-treated trypsin (1:50 w/w) for 20 h at room temperature. The tryptic peptides were separated by high-performance liquid chromatography on a Vydac C18 column. Sequences were determined using a Hewlett Packard G1000A, following the procedure standardized by the manufacturer.

## MEASUREMENT AND ANALYSIS OF OXYGEN BINDING ISOTHERMS

Oxygen equilibria were measured with the dilution method of Dolman and Gill (1978), using protein concentrations near 20 mg/ml. The temperature of the experiments was controlled to an accuracy of 0.05°C with a Lauda RMS bath. The changes in optical density were monitored to an accuracy of $10^{-4}$ OD units with an AVIV 14DS spectrophotometer at 438 nm. All experiments were performed in 0.1 M borate buffer at pH 9.0. The initial content of ferric hemoglobin was below 1%. The absorption spectra of the samples before and after the experiment were superimposable, indicating minimal methemoglobin formation during the experiments. Isotherms were performed at six to seven different temperatures in the interval 15°-37°C. Measurements of pH were performed on solutions brought to the desired temperature. The pH of borate buffer was negligibly sensitive to temperature.

For estimating the overall standard constants $\beta_{i,25}$ at each step of oxygenation and their overall standard enthalpy, ${}^{\beta}H_i$, at the reference temperature (25°C), the isotherms obtained at the various temperatures were simultaneously (globally) fit to the binding polynomial, $P$:

$$P = 1 + \Sigma_i \beta_{i,25} F_{i,T} X^i \qquad i = 1 \text{ to } 4 \tag{1}$$

where

$$F_{i,T} = \exp[({}^{\beta}H_i/R)(1/298.2 - 1/T)] \tag{2}$$

is the temperature correction factor for the temperature $T$, 298.2 is the reference temperature in Kelvin, $R$ is the gas constant, and $X$ is the ligand activity, in this case, the partial pressure of oxygen, $PO_2$.

The fit to the individual isotherms was done using

$$\Delta\Theta = \Delta(\mathrm{d} \ln P/4\mathrm{d} \ln X) \tag{3}$$

where $\Delta\Theta$ is the increment of fractional saturation of hemoglobin with oxygen at each step of the titration in the Gill's cell apparatus (Dolman and Gill, 1978).

The standard enthalpies, $\Delta H_i$, of the individual intrinsic constants were computed from the overall standard enthalpies using

$$^{\beta}H_i = \Sigma_i \Delta H_i \qquad i = 1 \text{ to } 4 \tag{4}$$

The standard intrinsic binding constants $K_{i,25}$ at 25°C were obtained from

$$K_{i,25} = i\beta_{i,25}/(5 - i)\beta_{i-1,25} \qquad i = 1 \text{ to } 4 \tag{5}$$

The median ligand activities at 25°C were computed from

$$P_{m,25} = (\beta_{4,25})^{-0.25}. \tag{6}$$

The standard Gibbs free energy changes were obtained using

$$\Delta G_{i,25} = -RT \ln K_{i,25} \qquad i = 1 \text{ to } 4 \tag{7}$$

The entropy changes were estimated from

$$\Delta G_{i,25} = \Delta H_i - T\Delta S_{i,25} \qquad i = 1 \text{ to } 4 \tag{8}$$

Computer analyses were performed using a nonlinear least-squares procedure described by Gill et al. (1987).

## RESULTS

### Position of the cross-linking bridge

Figs. 2 and 3 show a comparison of the tryptic peptide maps of adipoyl cross-linked and normal $\beta$-subunits. The chemical substitution produced the disappearance of tryptic peptides 9 and 10, as expected from acylation of the lysine at $\beta$82, at the end of peptide 9 (Table 1). It also shows that in the map of the cross-linked subunits, a new peak appeared that contained the combined peptide 9 + 10. Identical maps were obtained from the $\beta$-subunits of sebacoyl-Hb. These analyses proved that both chemically modified hemoglobins were intramolecularly cross-linked between the $\beta$82 lysyl residues, across the respective $\beta$-cleft.

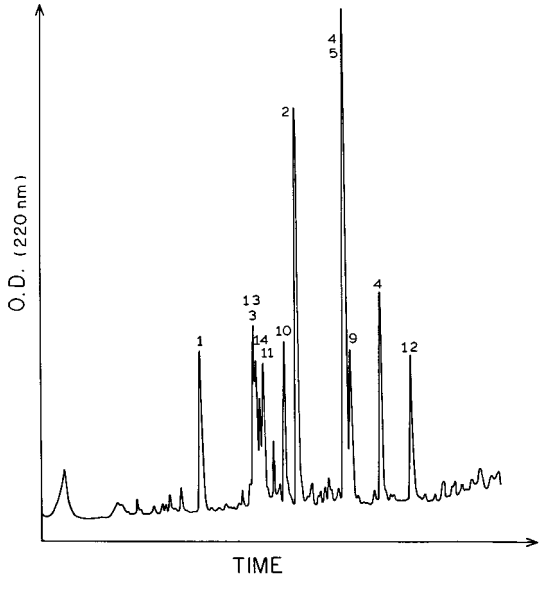


FIGURE 2 Peptide map of the $\beta$-subunits of normal hemoglobin.

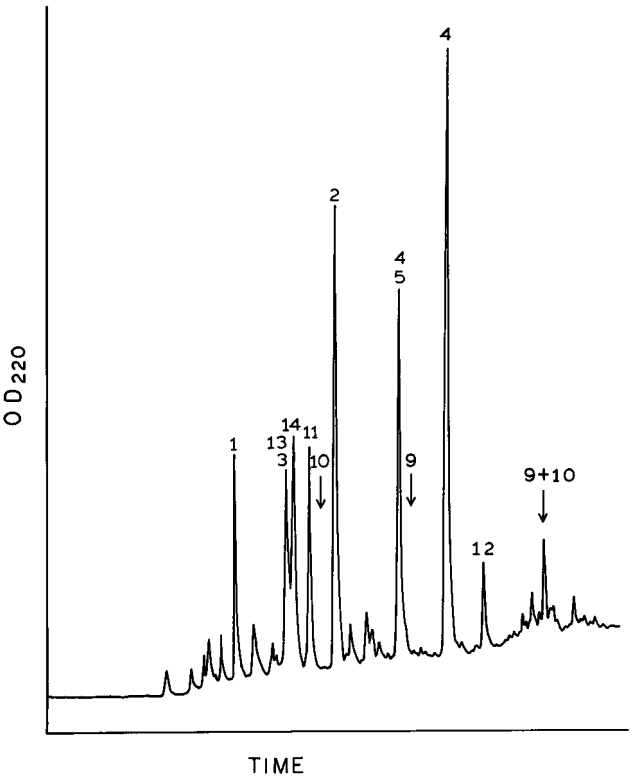


FIGURE 3 Peptide map of the cross-linked $\beta$-subunits of adipoyl-Hb. In comparison to that of normal $\beta$-subunits, note the absence of peptides 9 and 10 and the presence of a new fraction, which includes the combined peptide 9 + 10. The peptide map of the cross-linked $\beta$-subunits of sebacoyl-Hb were identical.

**TABLE 1 Tryptic fragments of human hemoglobin $\beta$-chain**

| Fragment | Residues | Sequence |
|---|---|---|
| 1 | 1–8 | VHLTPEEK |
| 2 | 9–17 | SAVTALWGK |
| 3 | 18–30 | VNVDEVGGEALGR |
| 4 | 31–40 | LLVVYPWTQR |
| 5 | 41–59 | FFESFGDLSTPDAVMGNPK |
| 6 | 60–61 | VK |
| 7 | 62–65 | AHGK |
| 8 | 66 | K |
| 9 | 67–82 | VLGAFSDGLAHLDNLK ← Lys 82 |
| 10 | 83–95 | GTFATLSELHCDK |
| 11 | 96–104 | LHVDPENFR |
| 12 | 105–120 | LLGNVLVCVLAHHFGK |
| 13 | 121–132 | EFTPPVQAAYQK |
| 14 | 133–144 | VVAGVANALAHK |
| 15 | 145–146 | YH |

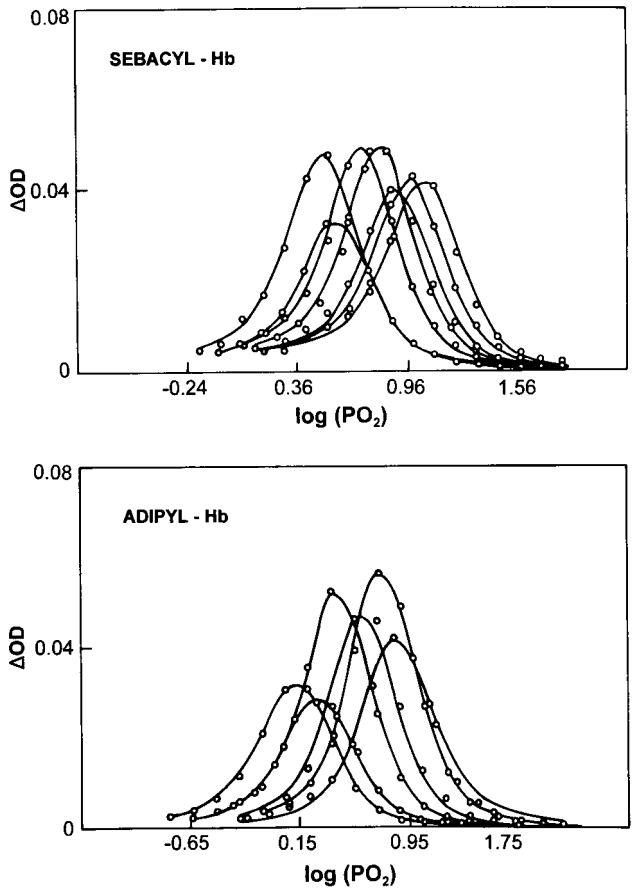


FIGURE 4 Binding isotherms of adipoyl-Hb and sebacoyl-Hb in 0.1 M borate at pH 9, at temperatures between 15° and 37° C. The interpolating lines are the computer fits of the global analyses.

## Oxygen binding isotherms and thermodynamic parameters

All of these measurements were conducted at pH 9.0 in 0.1 M borate buffer. We have already reported the oxygen binding isotherms at various temperatures of normal human hemoglobin, fumaryl cross-linked hemoglobin, and bovine hemoglobin (Bucci et al., 1991, 1993). In Fig. 4 we show the individual binding isotherms of adipoyl-, and sebacoyl-cross-linked hemoglobins, interpolated by the fits of the respective global analyses. The standard parameters at 25° were recovered from global analyses. Local (i.e., from individual isotherms) and global (i.e., from simultaneous analyses of all individual isotherms) $\beta_{i,T}$ values are listed in Tables 2 and 3. The restrictions imposed by the simultaneous analyses of independent data sets sharing the standard common parameters at 25°C allowed the standard deviation of the recovered quantities to fall very rapidly. In fact, the standard deviation of the estimated values for the common parameters progressively decreased with the inclusion in the analyses of additional independent isotherms, obtained at different temperatures. The tables show that the standard deviations of the local parameters values, in some case up to 300%, decreased to 1–3% in the simultaneous global analyses. The thermodynamic parameters listed in Tables 4 and 5 for adipoyl-Hb (hemoglobin cross-linked with an adipoyl residue, 6 carbons) and sebacoyl-Hb were recovered using Eqs. 5–8. The van't Hoff plots of the temperature-dependent $P_{m,T}$ values of adipoyl-Hb and sebacoyl-Hb, respectively, are shown in Fig. 5. The interpolating lines were computed using the average enthalpies obtained from the global analysis data in Tables 4 and 5 and show a very good correspondence between global and local values of the $P_{m,T}$ values of the two proteins. Furthermore, using the data reported in Tables 2–5, we compared the values of the intrinsic constants $K_i$ recovered either from local or from global analyses. This is shown in Figs. 6 and 7, where the symbols are for the values of intrinsic binding constants recovered from local analyses, and the continuous lines represent the values recovered from global analyses. Clearly, there is a good correspondence between the two sets of parameters. The dashed lines in the same figures were obtained from global analyses, where the enthalpies of the subsequent oxygenation steps were fixed to the average values obtained from the van't Hoff plots of the $P_m$ shown in Fig. 5. The dashed lines clearly fail to fit the data. In many instances, the inevitably positive slopes produced by the fixed average enthalpies do not match the negative trends of the data that unequivocally show the presence of an entropy-driven ligation step. Moreover, in Figs. 8 and 9, we compare the residual distributions produced by global analyses, using either floating or fixed average enthalpies, respectively. Reflecting the inconsistencies shown in Figs. 6 and 7, the sum of square residuals increases more than three times when the fixed average enthalpies are used. Furthermore, they resulted in systematic distortion of the distributions, indicating the failure of the average enthalpies to

**TABLE 2 Overall Adair constants at each oxygenation step of adipoyl-Hb**

| Temp | 18.3°C | | 21.6°C | | 25.0°C | | 29.0°C | | 33.3°C | | 36.8°C | | 25.0°C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L | G | L | G | L | G | L | G | L | G | L | G | G |
| $\beta_1$ torr$^{-1}$ | 0.91 ± 18% | 0.673 | 0.300 ± 31% | 0.475 | 0.413 ± 9% | 0.341 | 0.172 ± 22% | 0.233 | 0.133 ± 14% | 0.156 | 0.21 ± 16% | 0.114 | 0.341 |
| $\beta_2$ torr$^{-2}$ | 0.62 ± 15% | 0.570 | 0.310 ± 28% | 0.232 | 0.053 ± 21% | 0.098 | 0.0094 ± 74% | 0.037 | 0.0136 ± 20% | 0.013 | 0.014 ± 25% | 0.006 | 0.098 |
| $\beta_3$ torr$^{-3}$ | 0.016 ± 270% | 0.002 | 0.012 ± 310% | 0.002 | 0.022 ± 19% | 0.002 | 0.0065 ± 23% | 0.002 | 0.00184 ± 21% | 0.002 | 0.0006 ± 67% | 0.002 | 0.002 |
| $\beta_4$ torr$^{-4}$ | 0.523 ± 6% | 0.445 | 0.083 ± 12% | 0.100 | 0.024 ± 4% | 0.024 | 0.0024 ± 1% | 0.005 | 0.00086 ± 3% | 0.00084 | 0.00059 ± 7% | 0.000021 | 0.024 |

The data were recovered using Eq. 1 and were obtained either by local (L) analyses of single isotherms or by global (G), i.e., simultaneous, analyses of all isotherms. The standard deviations are given in as a percentage of the estimated values. For the global analyses, the standard deviation was less than 3% of the estimated value.

**TABLE 3 Overall Adair constants at each oxygenation step of sebacoyl-Hb**

| Temp | 18.3°C | | 20.6°C | | 25.1°C | | 28.4°C | | 31.65°C | | 34.0°C | | 36.8°C | | 25.0°C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L | G | L | G | L | G | L | G | L | G | L | G | L | G | G |
| $\beta_1$ torr$^{-1}$ | 1.027 ± 34% | 1.376 | 1.589 ± 13% | 1.163 | 1.05 ± 12% | 0.842 | 0.714 ± 6% | 0.669 | 0.556 ± 32% | 0.535 | 0.443 ± 41% | 0.457 | 0.385 ± 38% | 0.38 | 0.848 |
| $\beta_2$ torr$^{-2}$ | 0.071 ± 111% | 0.014 | 0.007 ± 256% | 0.013 | 0.0002 ± 250% | 0.012 | 0.0102 ± 196% | 0.011 | 0.0169 ± 70% | 0.010 | 0.005 ± 138% | 0.010 | 0.0075 ± 67% | 0.009 | 0.012 |
| $\beta_3$ torr$^{-3}$ | 0.021 ± 37% | 0.165 | 0.093 ± 6% | 0.080 | 0.025 ± 5% | 0.020 | 0.0095 ± 9% | 0.008 | 0.0037 ± 62% | 0.003 | 0.00196 ± 51% | 0.002 | 0.0009 ± 67% | 0.0007 | 0.021 |
| $\beta_4$ torr$^{-4}$ | 0.0716 ± 1% | 0.075 | 0.0527 ± 2% | 0.034 | 0.0087 ± 2% | 0.008 | 0.0024 ± 3% | 0.003 | 0.00126 ± 27% | 0.001 | 0.00039 ± 25% | 0.00046 | 0.0002 ± 30% | 0.0002 | 0.008 |

The data were recovered using Eq. 1 and were obtained either by local (L) analyses of single isotherms or by global (G), i.e., simultaneous, analyses of all isotherms. The standard deviations are given as a percentage of the estimated values. For the global analyses, the standard deviation was less than 3% of the estimated value.

match the temperature-dependent isotherms, especially at the low oxygen tensions.

## DISCUSSION

### Experimental conditions

Borate buffers have very small sensitivity to temperature. At pH 9.0, the Bohr effect of hemoglobin systems is practically finished, and the interaction with anions is minimal. Therefore we measured the behavior of "naked" tetrameric hemoglobin molecules. It should be stressed that, as previously reported (Bucci et al., 1991, 1993), under these conditions in the absence of chloride or phosphate ions, which are known to bind specifically to hemoglobin, especially at neutral pH, meaningful values of all Adair's constants are recovered, including the overall $\beta_3$ constant. This is shown in all of our data, those obtained in the past (Bucci et al., 1991, 1993) and those reported here. For measuring oxygen binding isotherms, we used the thin-layer dilution technique developed by Gill (Dolman and Gill, 1978). This allowed us to use high protein concentrations of 15 mg/ml or more, which increases the stability of the protein and, with non-cross-linked material, prevents distortions produced by significant amounts of dimers. We prefer this technology also because the measurement of the oxygen tension depends on a dilution factor that can be estimated with accuracy better than 1% and not on an oxygen electrode, which loses sensitivity at the low oxygen tensions necessary for measuring the high oxygen affinity of hemoglobin at alkaline pH.

### Global analysis of the data

The numerical analysis was based on the Adair formalism, as expressed in Eq. 1–3. To increase the accuracy of recovered parameters, we analyzed the data using global procedures. Global analysis implies the simultaneous optimization of independent data sets obtained under different conditions, in this case at different temperatures. The isotherms included in the global analysis shared as common parameters the values of the standard overall $\beta^0_i$ constants at 298.16 K and their respective enthalpy changes $\Delta H_i$ (Eq. 2). Contrary to local analysis, in which each isotherm is singularly analyzed, this imposed a constraint on the shared parameters, which had to satisfy simultaneously all of the independent isotherms included in the analysis. This drastically restricted the range of their estimation within limits common to all of the data sets. Furthermore, global analysis significantly increased the number of experimental data points used for the analysis. This resulted in better definitions of the recovered values. In a manuscript in preparation, we analyze the relevance of global analysis to oxygen binding isotherms. Those simulations show that three or more independent measurements must be simultaneously (globally) analyzed to obtain reliable information on the energetics of the intermediates of oxygenation. We generally use six or more isotherms for global analysis.

It should also be stressed that the "difference" step technology developed in the laboratory of Gill (Dolman and Gill, 1978) measures the oxygen-binding capacity of hemoglobin solutions, at stabilized equilibrium points. The binding capacity curves, by definition, start and end at zero

**TABLE 4 Standard thermodynamic parameters associated with the intrinsic affinity constants $K_{i,25}$, computed using Eq. 5 from the overall Adair constants of adipoyl-Hb**

| | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|
| $K$ (mM$^{-1}$) | 55.3 ± 0.8 | 124.4 ± 1.7 | 1989.8 ± 25.8 | 31179.5 ± 436.4 |
| $\Delta H$ (kcal/heme) | −14.2 ± 0.9 | −29.4 ± 2.1 | +51.7 ± 3.1 | −70.2 ± 4.3 |
| $\Delta G$ (kcal/heme) | −6.4 ± 0.4 | −6.9 ± 0.4 | −5.8 ± 0.3 | −10.2 ± 0.6 |
| $\Delta S$ (cal/deg/heme) | −26.1 ± 1.7 | −75.6 ± 5.5 | +192.9 ± 12.5 | −201.2 ± 13.3 |
| $T\Delta S$ (kcal/heme) | −7.8 ± 0.5 | −22.5 ± 1.7 | +57.5 ± 3.7 | −59.9 ± 3.9 |

The average enthalpy change is $\Delta H_{av} = -15.5$ kcal/heme.

**TABLE 5 Standard thermodynamic parameters associated with the intrinsic affinity constants $K_{i,25}$, computed using Eq. 5 from the overall Adair constants of sebacoyl-Hb**

| | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|
| $K$ ($mM^{-1}$) | 137.7 ± 1.7 | 6.1 ± 0.4 | 1705.1 ± 22.0 | 989.8 ± 8 |
| $\Delta H$ (kcal/heme) | −9.4 ± 0.5 | +11.4 ± 0.7 | −45.9 ± 1.8 | −1.7 ± 0.4 |
| $\Delta G$ (kcal/heme) | −7.0 ± 0.4 | −5.1 ± 0.3 | −8.5 ± 0.5 | −8.1 ± 0.5 |
| $\Delta S$ (cal/deg/heme) | −8.2 ± 0.4 | +55.6 ± 3.5 | −125.6 ± 6.3 | +21.6 ± 1.3 |
| $T\Delta S$ (kcal/heme) | −2.4 ± 0.1 | +16.6 ± 1.1 | −37.2 ± 1.9 | +6.4 ± 0.4 |

The average enthalpy change is $\Delta H_{av} = -11.4$ kcal/heme.

values. This avoids the necessity of determining the beginning and end points of the titrations, so critical to the numerical analysis and so difficult to obtain (Imai, 1990; Marden et al., 1989).

## Nonuniformity of enthalpy changes at the subsequent steps of oxygenation

Model-independent analyses of the intermediates of ligation in hemoglobin systems are based on two different statistics. The one proposed by Ackers and colleagues (Ackers formalism) for the assembly of tetrameric hemoglobins in different states of ligation, and the formalism of sequential binding (Adair formalism), as developed by Wyman and Gill (1990). The two statistics are difficult to compare, because they involve different distributions of intermediates. The Ackers formalism groups at the same energetic level species at different saturation with oxygen. The Adair formalisms strictly consider only species homogeneously saturated at the same level.

A common feature of the two formalisms is the presence of entropy-driven ligation steps, responsible for heat absorption. Atha and Ackers already noticed in 1974 that calorimetric data could be explained by a discontinuous release of heat at subsequent steps of oxygenation of human hemoglobin (Atha and Ackers, 1974). Imai in 1979 (Imai, 1979) noticed that at pH 9.0 the enthalpy change at the third step of oxygenation of human hemoglobin is sharply less exothermic, approaching a zero value, as in our data. Notably, the Imai data were obtained with an Imai cell (Imai et al., 1970) and not with a Gill cell (Dolman and Gill, 1978), as in our measurements. Other papers originating from the

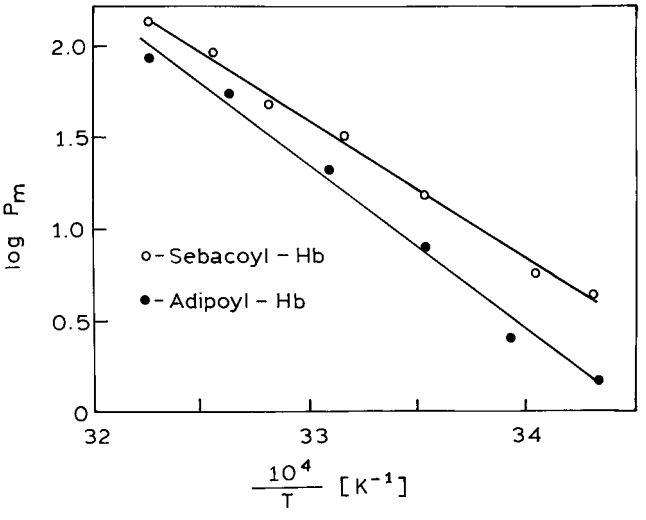


FIGURE 5 Van t'Hoff plots of the respective $P_{m,T}$ values of adipoyl-Hb and sebacoyl-Hb. The symbols are data obtained from single isotherm analyses. The continuous lines were obtained using the average $\Delta H$ values obtained from global analyses.

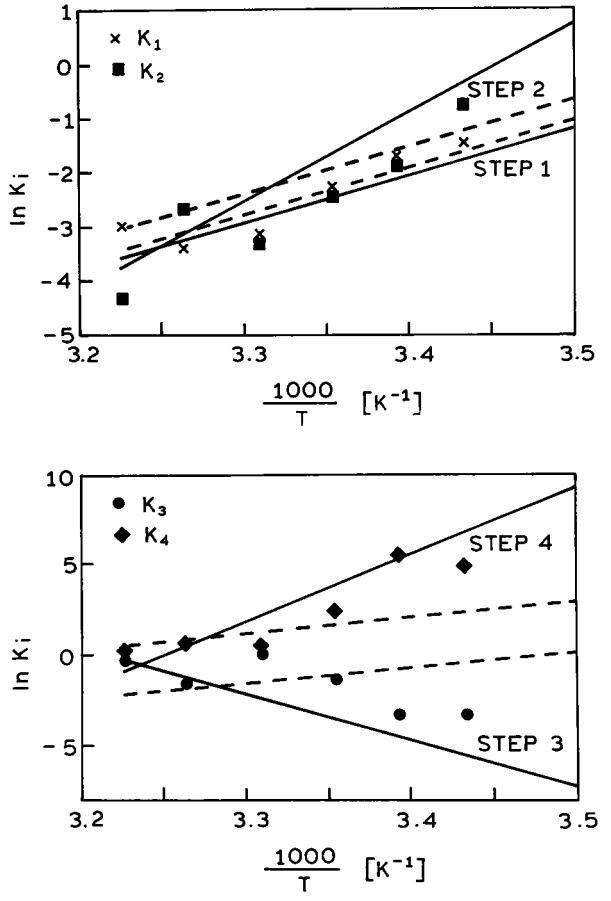


FIGURE 6 Temperature dependence of the intrinsic affinity constant $K_i$ at each step of oxygenation of adipoyl-Hb. The constants were computed from the overall Adair's constants, using Eq. 5. The symbols are the local data, obtained from single isotherm analysis, and the continuous lines were obtained using the enthalpy values obtained from global analyses of the isotherms. The dashed lines were obtained (with global analyses) assuming a constant enthalpy change at each oxygenation step equal to the average value obtained from the van t'Hoff plots in Fig. 5.

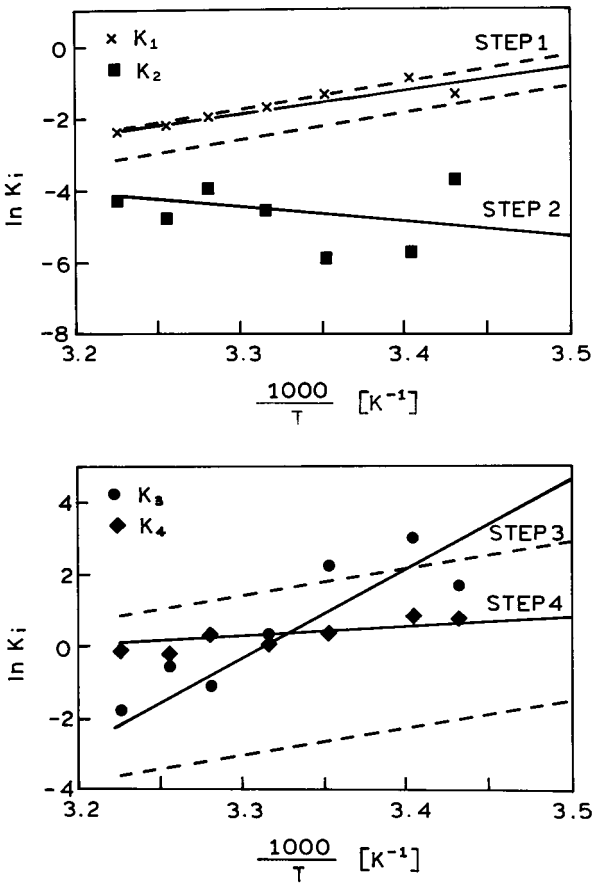


FIGURE 7 Temperature dependence of the intrinsic affinity constant $K_i$ at each step of oxygenation of sebacoyl-Hb. The constants were computed from the overall Adair's constants, using Eq. 5. The symbols are the local data, obtained from single isotherm analysis, and the continuous lines were obtained using the enthalpy values obtained from global analyses of the isotherms. The dashed lines were obtained (with global analyses) assuming a constant enthalpy change at each oxygenation step, equal to the average value obtained from the van t'Hoff plots in Fig. 5.

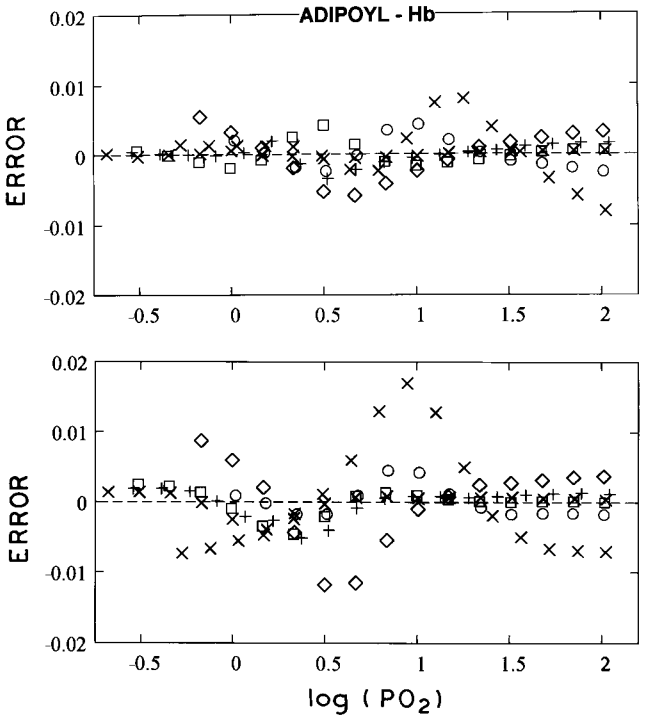


FIGURE 8 Error distribution for the global analysis of the isotherms of adipoyl-Hb. The upper panel is for the analysis in which the enthalpic parameters were left to float. The lower panel is for the analysis in which the enthalpies of all oxygenation steps were fixed at the average value obtained from Fig. 5. The average square errors are 0.003 and 0.009, respectively.

laboratory of Ackers (Mills et al., 1979; Chu et al., 1984) report calorimetric and van t'Hoff data on subunit assembly of hemoglobin. They confirm that the enthalpy of ligation depends on the extent of fractional saturation of the systems with ligands. In particular, a figure (Fig. 3) of Mills et al. (1979) shows a negative slope for the temperature dependence of the free energy of assembly of triply ligated hemoglobin tetramers, indicating an entropy-driven event. The presence of entropy-driven assembly reactions is also reported by Chu et al. (1984) and Huang and Ackers (1995).

The data presented here cannot be explained by a uniform distribution of heat. The constraint of a uniform heat distribution increased three to four times the overall sum of square residuals of the fits, resulting in nonrandom residual distributions. Most important, as shown in Figs. 6 and 7, the negative trend of the slopes of the data obtained by local analyses did not correspond to the positive slopes characteristic of a uniform heat distribution. In other words, uniform heat distribution would propose a temperature dependence of the intrinsic constants in some cases totally opposite that indicated by the experimental findings.

It is important that similar data have been obtained by direct calorimetry. Table 6 clearly shows the quantitative and qualitative consistency of van t'Hoff and calorimetric data obtained independently in different laboratories and under different buffer conditions, namely borate at pH 9.0 (Bucci et al., 1991, 1993) and phosphate at pH 7.4 (Johnson et al., 1992, 1995). Their consistency is striking. It is highly improbable that identical artifacts are present in totally different experimental approaches like optical and calorimetric measurements.

It should be stressed that the values of the positive and negative enthalpies are in some cases very large, and become even larger when they are corrected for the enthalpy of the reaction of oxygen with the heme iron, estimated at −14,000 cal, as shown in Table 7. This suggests that significant conformational fluctuations are responsible for the enthalpy/entropy variations.

It is important to notice that nonuniform heat distribution has been found in various natural and modified hemoglobins. They include human hemoglobin, natural and cross-linked with different dicarboxylic acid residues, including fumaric, adipic, and sebacic acid. They also include bovine and fallow deer (dama-dama) hemoglobins, indicating the presence of this characteristic across different species (Bucci et al., 1991, 1993; Johnson et al., 1992, 1995).

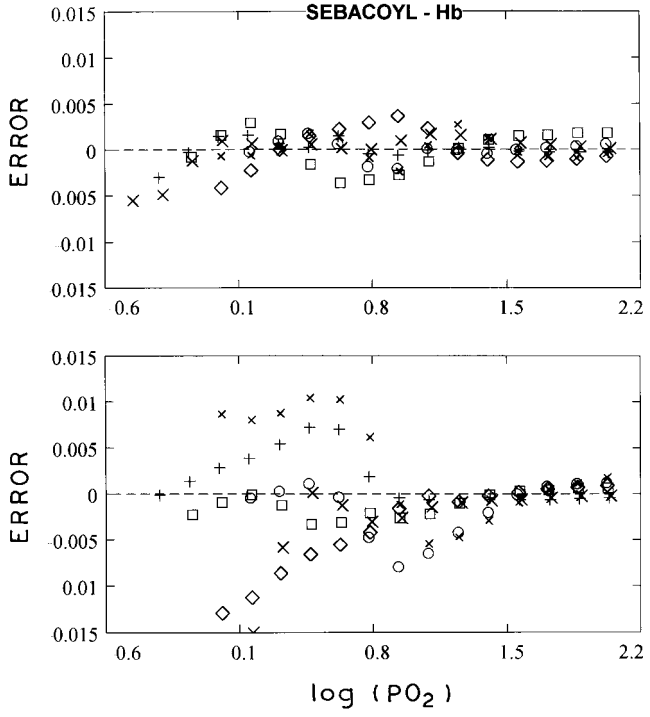


FIGURE 9 Error distribution for the global analysis of the isotherms of sebacoyl-Hb. The upper panel is for the analysis in which the enthalpic parameters were left to float. The lower panel is for the analyses in which the enthalpies of all oxygenation steps were fixed to the average value obtained from Fig. 5. The average square errors are 0.002 and 0.008, respectively.

It appears that in the Adair statistics, the presence of entropy-driven steps of oxygenation is a common characteristic of all allosteric hemoglobins investigated so far.

## Allosteric relevance of the data

The separation of enthalpy and entropy contributions to the free energy of oxygen binding of intermediate forms reveals a new aspect of the allosteric phenomena in the system. Since the publication in 1965 of the "plausible" model of Monod et al. (1965), the attention of investigators has been focused on the so-called switch point along the oxygenation pathway, the point at which there is a molecular transition from the T state to the R state. The question was apparently settled years before when Forbes and Roughton (1931) stated that the binding free energy decrease, responsible for the cooperativity, occurs at the fourth step of ligation, whereas for the first three steps, the binding free energy change remains very near the same level. This observation was never challenged. Table 8 confirms the findings of Forbes and Roughton. They show that for all of the systems reported here, the standard free energies of binding at the subsequent steps of ligation abruptly decrease by at least 2 kcal at the third or fourth step. (Sebacoyl-Hb is the only one whose critical decrease in binding free energy occurs at the third step of ligation.). Fumaryl-Hb (hemoglobin cross-linked with a fumaryl residue, four carbons), which is practically a nonallosteric system, does not show significant changes of the binding affinities at subsequent steps. Now it appears that the constant free energy change of the first steps is due to compensating changes in entropy and enthalpy, which mask entropy-driven steps.

The data suggest that the oxygen affinity and cooperativity index of all systems are strictly correlated with the extent of heat absorption, i.e., positive enthalpy changes, along the pathway of oxygenation. Fumaryl-Hb, with the least positive excess enthalpy, has the highest affinity and the lowest cooperativity index. Sebacoyl-Hb, with positive enthalpy excess at three oxygenation steps, has the lowest affinity and the next highest cooperativity. Adipoyl-Hb, with the largest positive enthalpy excess at the third step of oxygenation, has the next lowest oxygen affinity and the highest cooperativity.

It may also be noted that in all systems, except for the overconstrained fumaryl-Hb, the transition between the two levels of binding free energy shown in Table 7 is always preceded by a positive excess enthalpy (Table 7) and entropy change (Table 9), in which the system absorbs heat

**TABLE 6 Standard enthalpy changes at 25°C at subsequent steps of oxygenation, maximum value of the cooperativity index, and median ligand affinity of different hemoglobin systems, measured either by direct calorimetry (Cal) [11,12], or by van t'Hoff procedures [9,10]**

| Hemoglobin Species | $\Delta H_1$ (kcal/heme) | $\Delta H_2$ (kcal/heme) | $\Delta H_3$ (kcal/heme) | $\Delta H_4$ (kcal/heme) | $n$ | $P_{m,25}$ (torr) |
|---|---|---|---|---|---|---|
| Human* Cal | −10.3 | −16.8 | −0.7 | −20.3 | 2.7 | 7.5 |
| Human[#] | −12.6 | −27.7 | 0.5 | −22.9 | 2.3 | 1.4 |
| Bovine* Cal | −6.7 | −31.8 | 20.2 | −16.9 | 2.8 | 17.5 |
| Bovine[#] | 0.8 | −35.8 | 23.0 | −20.5 | 2.4 | 3.7 |
| Dama[§] Cal | −4.8 | −26.3 | 2.8 | −2.1 | 2.4 | 20.9 |
| Adipoyl[#] | −14.2 | −29.4 | 51.6 | −70.2 | 2.7 | 2.5 |
| Sebacoyl[#] | −9.4 | 11.4 | −45.9 | −1.7 | 2.3 | 3.3 |
| Fumaryl[#] | −24.2 | −21.0 | −12.2 | −18.8 | 1.4 | 1.4 |

Calorimetric data have a higher value of $n$ and $P_{m,25}$ than the van t'Hoff data because they were obtained at neutral pH and at pH 9.0, respectively.
*0.1 M phosphate buffer at pH 7.6, 1 mM EDTA, in the presence of a reducing enzyme [11].
[#]0.1 M borate buffer at pH 9 [9,10].
[§]0.1 M phosphate buffer at pH 7.4, in the presence of a reducing enzyme [12].

**TABLE 7 Excess enthalpy changes at 25°C at subsequent steps of oxygenation, maximum value of the cooperativity index and median ligand affinity of different hemoglobin systems, measured either by direct calorimetry (Cal) [11,12], or by van t'Hoff procedures [9,10]**

| Hemoglobin species | $\Delta\Delta H_1$ (kcal/heme) | $\Delta\Delta H_2$ (kcal/heme) | $\Delta\Delta H_3$ (kcal/heme) | $\Delta\Delta H_4$ (kcal/heme) | $n$ | $P_{m,25}$ (torr) |
|---|---|---|---|---|---|---|
| Human* Cal | 3.7 | −2.8 | 13.7 | −6.3 | 2.7 | 7.5 |
| Human[#] | 0.4 | −13.7 | 14.5 | −8.9 | 2.3 | 1.4 |
| Bovine* Cal | 7.3 | −17.8 | 34.2 | −2.9 | 2.8 | 17.5 |
| Bovine[#] | 14.8 | −21.8 | 37.1 | −6.5 | 2.4 | 3.7 |
| Dama[§] Cal | 9.2 | −12.3 | 16.8 | 11.9 | 2.4 | 20.9 |
| Adipoyl[#] | −0.22 | −15.4 | 65.6 | −56.1 | 2.7 | 2.5 |
| Sebacoyl[#] | 4.5 | 25.4 | −31.9 | 12.3 | 2.3 | 3.3 |
| Fumaryl[#] | −10.2 | −7.1 | 2.1 | −4.8 | 1.4 | 1.4 |

Calorimetric data have a higher value of $n$ and $P_{m,25}$ than the van t'Hoff data because they were obtained at neutral pH and at pH 9.0, respectively.
*0.1 M phosphate buffer at pH 7.6, 1 mM EDTA, in the presence of a reducing enzyme [11].
[#]0.1 M borate buffer at pH 9 [8,9].
[§]0.1 M phosphate buffer at pH 7.4, in the presence of a reducing enzyme [12].

and becomes more disordered. There is a rough correlation between the size of the positive changes of the two parameters and the final enhancements of the $\Delta G$ values. Adipoyl-Hb shows a positive excess enthalpy change of 70 kcal and a positive entropy change of 192 eu at the third step of ligation, before a jump of −5 kcal of its free energy of ligation at the fourth step.

## Informational content of the data

The thermodynamics of oxygen binding by either natural or cross-linked hemoglobins indicate that

1. The entropy-driven steps, with absorption of heat, belong to the intermediates of ligation.
2. When absorption of heat is inhibited by cross-linking (with fumaryl residues), the oxygen affinity of the system increases and the cooperativity index becomes very small.
3. In the subsequent binding steps, the negative increase in the free energy of oxygen binding of at least 2 kcal, which produces a cooperativity index of 2.0 or more, is always preceded by a distinct absorption of heat.
4. In all hemoglobins, all subsequent binding steps are characterized by compensatory changes in entropy and enthalpy, except the last one, when the free energy of binding drastically decreases.
5. The nonuniform enthalpy changes are differently distributed in the intermediates of ligation of either different species or differently cross-linked hemoglobins.

**TABLE 8 Standard free energy values at 25°C at subsequent steps of oxygenation of the various hemoglobin systems**

| Hemoglobin species | $\Delta G_1$ (kcal/heme) | $\Delta G_2$ (kcal/heme) | $\Delta G_3$ (kcal/heme) | $\Delta G_4$ (kcal/heme) |
|---|---|---|---|---|
| Human* | −6.9 | −7.2 | −7.6 | −8.8 |
| Human (Cal.)[#] | −5.7 | −6.2 | −6.5 | −8.5 |
| Bovine* | −6.4 | −6.2 | −7.1 | −8.0 |
| Bovine (Cal.)[#] | −5.5 | −4.4 | −7.1 | −7.9 |
| Dama (Cal.)[§] | −5.8 | −4.5 | −6.8 | −7.3 |
| Fumaryl[#] | −8.2 | −7.6 | −8.3 | −8.4 |
| Adipoyl[#] | −6.4 | −6.9 | −5.8 | −10.2 |
| Sebacoyl[#] | −7.0 | −5.1 | −8.5 | −8.1 |

*[#§]See Table 7 for meaning of symbols.

These observations suggest that absorption of heat is a component of the allosteric properties of hemoglobin. It is as if a maximum disorder is to be obtained before the system relaxes into a fully oxygenated molecule. Only at the fourth step of oxygenation are enthalpy and entropy changes not compensatory, suggesting that binding cooperativity results from an imbalance of enthalpy and entropy changes.

The large excess of enthalpies shown in Table 7 suggests that the nonuniform release of heat of the intermediates of oxygenation is supported by significant conformational changes.

Regarding possible conformational changes that may explain the phenomena reported above, several conformations are available to hemoglobin, whose interplay may be responsible for the entropy-driven steps. To the classic T and R structures (Fermi et al., 1984; Shanaan, 1983) we can add the crystal structures of R2 (Silva et al., 1992) and of hemoglobins partially oxygenated in either the $\alpha$- or the $\beta$-subunits (Liddington et al., 1992). In our laboratory we have resolved the crystal structure of unliganded sebacoyl-Hb partially oxidized to ferric form in the $\alpha$-subunits (Xinhua et al., in press). The atomic coordinates of fully oxygenated T are also available (Liddington et al., 1988). In all of these forms, ligands were accommodated by displacements of heme-pocket residues. The crystal structure of a few cross-linked liganded hemoglobins has been reported. Trimesil cross-linked Hbs appeared to have intermediate tertiary conformations between the T and R structures (Schumacher et al., 1995). Liganded hemoglobins cross-linked with 4-carboxycinnamic acid and 2,6-naphthalene dicarboxylic acid also appeared to have new tertiary structures intermediate between R and R2 (Schumacher et al., 1997). Also described is the "r" structure of deoxyhemo-

**TABLE 9 Standard entropy changes at 25°C at subsequent steps of oxygenation of the various hemoglobin systems**

| Hemoglobin species | $\Delta S_1$ (eu/heme) | $\Delta S_2$ (eu/heme) | $\Delta S_3$ (eu/heme) | $\Delta S_4$ (eu/heme) |
|---|---|---|---|---|
| Human* | −19 | −68 | 27 | −47 |
| Human (Cal.)# | −15 | −35 | 20 | −40 |
| Bovine* | 24 | −99 | 101 | −39 |
| Bovine (Cal.)# | −4 | −92 | 92 | −36 |
| Dama (Cal.)§ | 3 | −73 | 32 | 17 |
| Fumaryl# | −53 | −45 | −13 | −25 |
| Adipoyl# | −26 | −75 | 192 | −201 |
| Sebacoyl# | −8 | 55 | −125 | 21 |

*#§See Table 7 for meaning of symbols.

globin trapped in a high-affinity state, which does not seem to be within the envelope of the T-R transformations (Wilson et al., 1996). They all have been proposed to be representative of intermediates of oxygenation. We may also add that the absence of covalent bonds between the subunits of hemoglobin allows formation of dimers, and even of monomers (Gryczynski et al., 1997; Pin et al., 1990). The allosteric relevance of dimer formation is well described by Ackers et al. (1992). Ackers and Smith (1987) and Jayaraman and Spiro (1995) report the presence of a third cooperative state of hemoglobin besides the T and R conformations. The hydration shell of hemoglobin may also participate in the entropy/enthalpy changes.

It is interesting to note that modifications of the tertiary structure of hemoglobin do not necessarily produce modification of the subunit assembly. This adds to the notion that hemoglobin has a substantial degree of freedom in its hierarchical structures.

Colombo et al. (1992, 1994) report a larger amount of water to be "bound" by the liganded form of hemoglobin. Immobilization of water molecules in the hydration shell would liberate heat (negative enthalpy), and their release would absorb heat, thereby producing positive enthalpy changes.

It should be stressed that the description of new crystal structures, therefore obtained under equilibrium conditions, implies that those conformations are always available to the system. These are selected as part of the experimental environment and include pH, temperature, ionic strength, anionic effectors, presence of ligands, presence of intramolecular cross-links, and crystal forces. Furthermore, our data show that the distributions of heat release at the intermediates of oxygenation are different for the different species and for the different cross-links (Table 6). Still, all of these systems have a distinct oxygen binding cooperativity and a low oxygen affinity, lower than that of natural HbA, suggesting that the allosteric behavior of hemoglobin systems can be supported by several nonidentical conformational changes, selected either by amino acid composition in the different species or by the chemicals that cross-link the same hemoglobin—in our case, HbA.

All of the various hemoglobin structures mentioned above may participate, in principle, in these phenomena. At present there are not enough data that would allow specific assignment of the entropy-driven steps to any particular tertiary or quaternary conformation. The possibility cannot be excluded that the entropy-driven steps are related to novel conformations of hemoglobin still to be described.

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