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Articles

Effect of Intramolecular Cross-Links on the Enthalpy and Quaternary Structure of the Intermediates of Oxygenation of Human Hemoglobin†

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ABSTRACT: We have reported [Bucci, E., Fronticelli, C., & Grycznski, Z. (1991) *Biochemistry 30*, 3195–3199] that in human and bovine hemoglobins the release of heat at the subsequent steps of oxygenation is not constant. This is especially evident in the binding of the third O_2 molecule, which is an endothermic event. This phenomenon was attributed to peculiar conformations of the intermediates of oxygenation, not included in the fundamental R/T transition of the system. To test this hypothesis, we have explored the effect of conformational constraints on the thermodynamics of the intermediates of oxygenation. The assumption was that intramolecular constraints would stabilize the intermediates into conformations similar to the R and T forms reducing the variability of their enthalpies. We have analyzed the temperature dependence of the oxygen binding isotherms of human hemoglobin cross-linked either between the β82 or between the α99 lysines by bis(3,5-dibromosalicyl)fumarate. The measurements were performed at pH 9.0 in 0.1 M borate buffer in order to avoid thermal effects due to oxygen-linked binding of anions and protons. The data were analyzed singularly by local procedures and simultaneously using global procedures. The two cross-links had opposite effects. The cross-link between the β-subunits decreased while that between the α-subunits increased the endothermic behavior of the third step of oxygenation. Also, the cross-link between the β-subunits increased the fractional amount of the triligated species at intermediate stages of oxygenation, while that between the α-subunits decreased this quantity to hardly detectable values. These data are consistent with the hypothesis that the R/T transition in hemoglobin involves novel conformations not included in the R/T system. It is also speculated that the novel conformations of the intermediates of oxygenation is similar to the Y structure of hemoglobin Ypsilanti.

The cooperativity of ligand binding to hemoglobin tetramers is produced by a scarcity of the intermediate species of ligation between 0 and 4 ligands per molecule, which defies the statistics expected from random ligand binding to available hemes. The mechanism which regulates the statistics of oxygen binding is linked to the structure–function properties of the intermediates of ligation.

The scarcity of these intermediates is in an impediment to their investigation. Many attempts have been made for stabilizing partially ligated species, based either on the assumption that ferric subunits are identical, or very similar, to normal oxygenated chains or that subunits with non-iron metalloporphyrins could substitute for either liganded or

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unliganded normal chains (Bluogh et al., 1984; Miura et al., 1987; Simolo et al., 1986; Mawatori et al., 1987; Arnone et al., 1988; Ishimori et al., 1989; Luisi & Shibayama, 1989; Hofrichter et al., 1985; Waterman & Yonetani, 1970; Doyle et al., 1991). The limitation of this approach was that only symmetric hybrids were produced with either both α- or both β-subunits ferric or metal-substituted. The information so obtained suggested that these intermediates had conformational and functional properties very similar to or comprised between those of either the T or the R structures of the system.

Mixtures of ferric and liganded hemoglobins with symmetric ferrous–ferric hybrid molecules were also utilized for enriching the solutions of asymmetric ferrous–ferric hybrids. The dimer–tetramer dissociability and the kinetics of the reactions of these mixtures with ligands suggested the presence of more conformational species than the R and T structure alone (Ackers & Smith, 1987; Sharma et al., 1991; Berjis et al., 1990). Those data did not indicate whether the additional conformations were comprised between the R and T forms of the system.

Recently, Fowler et al. (1992) succeeded in isolating the triferric form of human hemoglobin cross-linked between the α99 lysines by acylation with a fumaryl residue. The kinetics of ligand binding of this species was ligand dependent, challenging the view that the triligated species is in the R form. A similar challenge was proposed earlier by Ferrone et al. (1985), using modulation excitation techniques.

Thus, there is increasing evidence that the two-state model cannot completely explain the thermodynamics of hemoglobin. Modifications of the two-state model have been proposed which include additional conformational intermediates (Ackers & Smith, 1987; Miura et al., 1984, 1987; Gill et al., 1987).

The third step of oxygenation is currently under scrutiny. Results published by Smith and Ackers (1985) show a critical conformational change in hemoglobin when the third ligand enters the molecule. Perutz (1990) suggests that the conformational switch of the system occurs at the third ligation step. Data from our laboratory (Fronticelli et al., 1993) indicate that the energetics of conformational changes produced by the βE11Val → Thr mutation is mostly confined to the third step of ligation. The queston is posed whether the third ligation produces a special conformation of the system.

In a recent paper (Bucci et al., 1991), we have shown that the enthalpy of oxygenation of human and bovine hemoglobins is not constant at each oxygenation step and that the discontinuity is especially evident in the endothermic behavior of the third step of oxygenation. This suggested the presence in the system of an unusual conformation of the triligated specie, not included in the R/T system. The large endothermic anomaly was consistent with a rearrangement of the quaternary structure producing substantial changes of the hydrophobicity of the surface of the protein.

We tested this hypothesis by introducing in the system constraints to the quaternary structure, which would impede conformational excursions of the protein outside of the R/T system.

Intramolecular cross-links of the β-subunits are obtained using the FBDA[1] reagent (Walder et al., 1980). In oxyhemoglobin, this reagent leaves a fumaryl bridge between the β82 lysines of the partner β-chains, which imposes constraints on the quaternary structure of the system. In human hemoglobin, introduction of this cross-link leaves in the system a cooperativity with a Hill parameter above 2.0 at neutral pH (Walder et al., 1980).

The same reagent in deoxyhemoglobin produces a cross-link between the α99 lysines, resulting in a hemoglobin with quasinormal cooperativity and a reduced oxygen affinity (Chattarjee et al., 1986). Also in this case, constraints are imposed on the quaternary structure of the system.

The β- and α-cross-linked human hemoglobin appeared to be the ideal system in which to study the influence of conformational constraints on the thermodynamics of the intermediate steps of oxygenation.

MATERIALS AND METHODS

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

The β-cross-linked derivative of human oxyhemoglobin (HbAβXL) was prepared by incubating a 6% solution of hemoglobin in 0.05 M Tris buffer at pH 7.0 with 0.7 mg/mL FBDA reagent for 60 min at 37 °C. The reaction was stopped by the addition of glycylglycine up to a concentration of 0.05 M for 30 min at 37 °C, followed by dialysis in the cold against 0.1 M glycine, overnight. Chromatographically, pure fractions were obtained using a Waters Delta-Prep 4000 preparative chromatograph with a 2.5 × 20 cm column packed with DEAE 650M Toyopearl resin (Supelco). The elution gradient was 0.015 M Tris buffer at pH 8.2 and 0.015 M Tris buffer at pH 7.5 in 0.2 M sodium acetate. Also, all buffers contained 0.5 mM EDTA.

Human hemoglobin cross-linked between the α99 lysines (HbAαXL) was a generous gift of the Letterman Army Institute of Research. The protein was further purified by column chromatography using the same gradient system described above.

Measurement and Analysis of Oxygen Binding Isotherms. Oxygen equilibria were measured with the dilution method of Dolman and Gill (1978), using protein concentrations near 30 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 density were monitored with accuracy to 10^{-5} 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 3%. The absorption spectra of the samples before and after the experiment were superimposable, indicating absence of methemoglobin formation during the experiments.

Measurements of pH were performed on solutions brought to the desired temperature. The pH of borate buffer was insensitive to temperature.

For each individual measurement, changes in optical absorbance upon stepwise changes of PO_2 were fit to

$$\Delta OD_i = \Delta OD_T \Delta\theta_i \quad (1)$$

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 using (Wyman, 1964)

$$\theta = d \ln P/4\, d \ln X \quad (2)$$

[1] Abbreviations: FBDA, fumarylbis(dibromo-Aspirin) = bis(3,5-dibromosalicyl)fumarate; HbAβXL, human hemoglobin cross-linked between the β-subunits; HbAαXL, human hemoglobin cross-linked between the α-subunits; HbA, human hemoglobin A; Tris, tris(hydroxymethyl)aminomethane.

where P is the binding polynomial

$$P = 1 + \sum_i \beta_i X^i \quad i = 1\text{–}4 \tag{3}$$

X is the free ligand concentration (i.e., PO_2 in mmHg), and β_i's are the overall affinity constants defined as

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

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

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

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

where T is the temperature of the various experiments in Kelvin, 298.2 is the reference temperature in Kelvin, and R is the gas constant. The standard enthalpies, $\Delta H_{25,i}$, of the individual intrinsic constants were computed from the overall standard enthalpies using

$$\beta H_{25,i} = \sum_i \Delta H_{25,i} \quad i = 1\text{–}4 \tag{6}$$

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

We computed the distributions, α_i, of the various intermediates upon titration with oxygen using

$$\alpha_i = \beta_i X^i / P \tag{7}$$

where P is the binding polynomial of eq 3.

RESULTS

Shibayama et al. (1991) report that their preparations of HbAβXL contained about 20% of an impurity, which was undetectable in chromatographic and electrophoretic analyses. Therefore, we conducted a very careful peptide analysis of our preparations of HbAβXL and, as reported in detail, we could not find any other chemical substitution besides the cross-link between the two β82 lysines. We concluded that if we had impurities, they were too small to be significative. The evidence is as follows.

Peptide Analysis of HbAβXL

Peptide Preparation and Methods. Prior to tryptic cleavage, 25–30-nmol samples of β-chain, purified from human HbA either before or after FBDA treatment, were *S*-pyridylethylated by dissolving in 0.25 M Tris-HCl, 1 mM EDTA, and 6 M urea (pH 8.5). To this was added 8 μL of a 10% aqueous solution of β-mercaptoethanol and, after stirring under nitrogen for 1 h, 2.5 μL of 4-vinylpyridine. The reaction took place at room temperature for 90 min under nitrogen. The sample was then dried using a Savant SpeedVac concentrator, redissolved in 300 μL of 70% HCOOH, and desalted by reverse-phase HPLC using a 4.6 × 250 mm Vydac 214TP54 C_4 column. The column had been previously equilibrated with 0.1% aqueous trifluoroacetic acid (TFA) as solvent A. Following injection of the sample and washout of salts and small molecules, the pyridylethylated protein was eluted with a 30-min gradient from solvent A to a 90:10 (v/v) mixture of acetonitrile and solvent A. The protein was detected by monitoring the absorbance of the eluent at 280 nm and was collected manually.

The pyridylethylated protein was dried as before, redissolved in 900 μL of 100 mM NH_4HCO_3 (pH 7.5), and tryptic digestion was performed overnight at 37 °C using a 1:50 weight ratio of trypsin to β-chain. The resulting clear solution was then directly injected onto a 4.6 × 250 mm Vydac 218TP54 C_{18} reverse-phase column for purification of the tryptic peptides. The column had been previously equilibrated with 0.1% aqueous TFA as solvent A and peptides were eluted with linear gradients of solvent B [0.1% TFA in a 9:1 (v/v) mixture of acetonitrile and water]. The gradients were as follows: 0–50% B over 60 min, followed by 50–100% B over 15 min. Peptides were detected by monitoring the absorbance of the eluent at 215 nm. Peptide-containing fractions were collected manually and used for amino acid analysis or sequencing. A Waters HPLC system equipped with a 1-mL sample injection loop, two Waters M510 pumps, a Waters model 490E multiwavelength detector and a Waters M680 gradient controller was used, at a flow rate of 1.0 mL/min, for both desalting and peptide purification.

Amino acid compositions were determined by the method of Heinrickson and Meredith (1984). Phenylthiocarbamylated acid hydrolysates of samples were prepared using a Waters "PICO-TAG" workstation. PTC-amino acids were analyzed by reverse-phase HPLC on a Waters "PICO-TAG" column, using the gradient elution system recommended by the manufacturer. Sequences were determined using an Applied Biosystems model 477A sequencer equipped with an on-line model 120A phenylthiohydantoin (PTH) analyzer. The PTH derivative of pyridylethylcysteine, when present, was easily identified.

Peptide Maps of HbA and HbAβXL. The α- and β-subunits of HbA and HbAβXl were isolated by reverse chromatography (Shelton et al., 1981). In HbAβXL only the β-subunits had a different elution time then in normal hemoglobin. Both chains gave single homogeneous peak in the elution profile. The HPLC profiles of tryptic digests of the β-chain purified from unmodified HbA (Figure 1a) and HbAβXL (Figure 1b) show several differences. Most noticeable are the disappearance of three peaks after FBDA treatment. Sequence analysis showed that one of these peaks, eluting at ~45 min, contained tryptic fragment 9 (residues 67–82, see Table I) and the other, eluting at ~36 min, contained fragment 10 (residues 83–95). The loss of fragments 9 and 10 is exactly what would be expected if Lys-82 were modified, since this would prevent tryptic cleavage at that residue. The third peak to disappear elutes at ~38 min, just prior to fragment 2. This peak, which does not always appear in tryptic peptide maps of the β-chain, was shown by sequence analyses to contain a low-yield mixture, mainly of peptides derived from autolysis of trypsin.

Although most of fragment 10 was recovered in the disappearing peak which eluted at ~36 min, this peptide was also represented in two other peaks prior to FBDA treatment. The first of these peaks eluted at ~42 min and contained a mixture of fragment 5 (residues 41–59) and the partial cleavage product fragment 10+11 (residues 83–104). After FBDA treatment, this peak decreased in size and was found to contain only fragment 5. (The recovery in this peak of a small portion of the total fragment 5 may have arisen from partial oxidation of Met-55). Another peak, eluting at ~54 min in HbA, contained fragment 12 (residues 105–120), and also fragment 10, in a molar ratio of ~9:1. A gap in the sequence at the

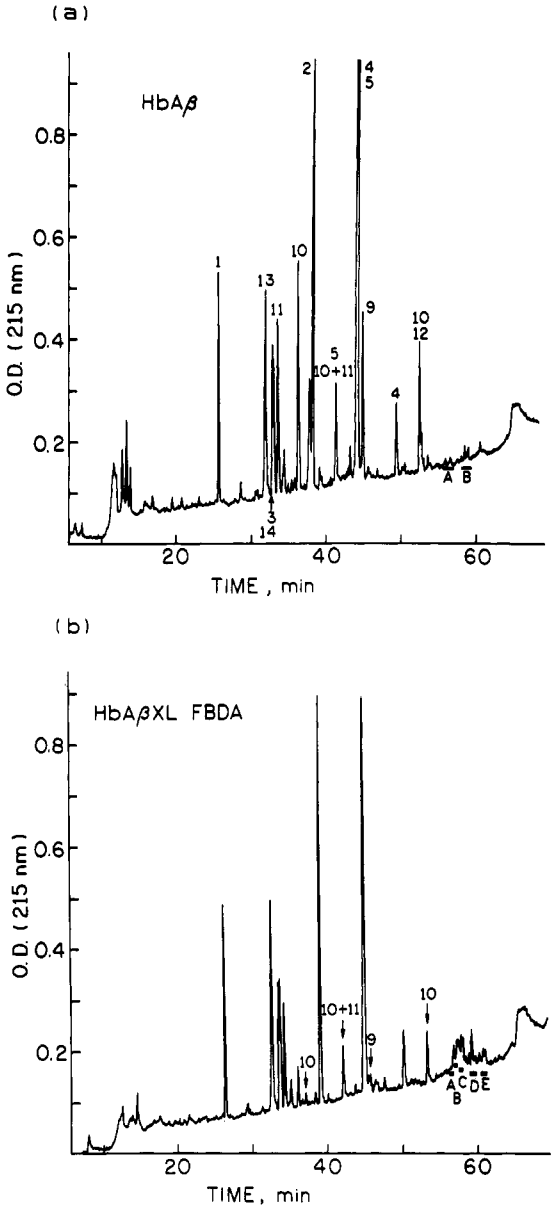

FIGURE 1: Tryptic peptide maps of the β-chain from unmodified (a) and FBDA-treated (b) human HbA. Following pyridylethylation of Cys thiol groups, the β-chain was digested with trypsin, and peptides were separated by reverse-phase HPLC using a Vydac 218TP54 column, with a gradient consisting of 0.1% trifluoroacetic acid and increasing concentration of acetonitrile. Peptides were collected manually and identified by amino acid analysis or sequencing. (See the text for experimental details.) The numbers shown in panel a correspond to the tryptic fragments of the β-chain, which are listed in Table I. The numbers, and accompanying arrows, shown in panel b identify the peptides which disappeared after FBDA treatment. Fractions A–E in panel b, but not A and B in panel a, indicated by short horizontal bars, contained cross-linked peptides which appeared after FBDA treatment in HbAβXL.

position of Cys-93 indicated that the fragment 10 in this peak may have been oxidized to its disulfide form. After FBDA treatment, this peak also decreased in size and was found to contain only fragment 12.

After FBDA treatment, a cluster of late-eluting small peaks, presumably containing cross-linked peptides, appeared. Fractions A–E from this cluster (see Figure 1b) were selected for sequence analyses, and in each case a mixture of two sequences was found. One of the sequences spanned fragments 9+10 (residues 67–95), with a gap in the position of Lys-82, as would be expected if this residue were modified by FBDA. The second sequence found throughout fractions A–E was identical to fragment 12 (residues 105–120), except that a gap occurred at the position of Cys-112. This suggests that fragment 12 was partially oxidized to its disulfide form, accounting for its appearance in these late fractions. The molar ratios of the two sequences in fractions A–E were variable and nonstoichiometric, so it is not likely that they represent peptides which are cross-linked to each other. Cys-112, and other residues in fragment 12, are too far away from Lys-82 to form cross-links in the native HbA structure. To further test this possibility, sequence analyses were performed on equivalent late-eluting fractions from the tryptic digest of unmodified β-chain (fractions A and B in Figure 1a). These fractions yielded a single major sequence: fragment 12 (residues 105–120), with a blank at the position of Cys-112. Therefore, fragment 9+10, with a modified Lys-82, is the only new peptide that appears after crosslinking.

Table I: Tryptic Fragments of Human Hemoglobin β-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
10	83–95	GTFATLSELHCDK
11	96–104	LHVDPENFR
12	105–120	LLGNVLVCVLAHHFGK
13	121–132	EFTPPVQAAYQK
14	133–144	VVAGVANALAHK
15	145–146	YH

The differences in the tryptic peptide maps shown in Figure 1a, and Figure 1b are thus solely attributable to modification of Lys-82, leading to the disappearance of fragments 9 and 10 and the appearance of a new fragment 9+10. Since the β-chain is covalently dimerized by FBDA, and the Lys-82 side chains of both β-subunits of native HbA are close together, fragment 9+10 is undoubtedly a cross-linked dimer connected at its two Lys-82 residues by a fumaryl group.

It has been suggested, on the basis of steric considerations (Shibayama et al., 1991), that FBDA treatment of native HbA could form cross-links between Lys-82 of one β-chain and either Val-1 or Lys-144 of the other β-chain. Our results show that this did not occur. First of all, the peptide peaks which appeared after cross-linking (fractions A–E in Figure 1b) did not contain any sequences which might include Lys-144. Cross-linking of Val-1 would have occurred at its α-amino group, resulting in a blocked N-terminus which could not be detected by sequence analysis. A comparison of Figure 1 panels a and b, however, clearly shows that Val-1 is not cross-linked, since the recovery of fragment 1 (residues 1–8) is not affected by FBDA treatment. Furthermore, five steps of sequence analyses on intact β-chain, both before and after cross-linking, gave comparable yields of the expected N-terminal sequence. This is consistent with steric considerations which indicate the distance between β82Lys and β1 Val to be too large to be spanned by a fumaryl residue.

Temperature Dependence of Oxygen Binding by HbAβXL and HbAαXL

Figure 2 shows the binding isotherms used for the global analyses of the two cross-linked hemoglobins. The continuous lines represent the fit to the data using equation 5. From

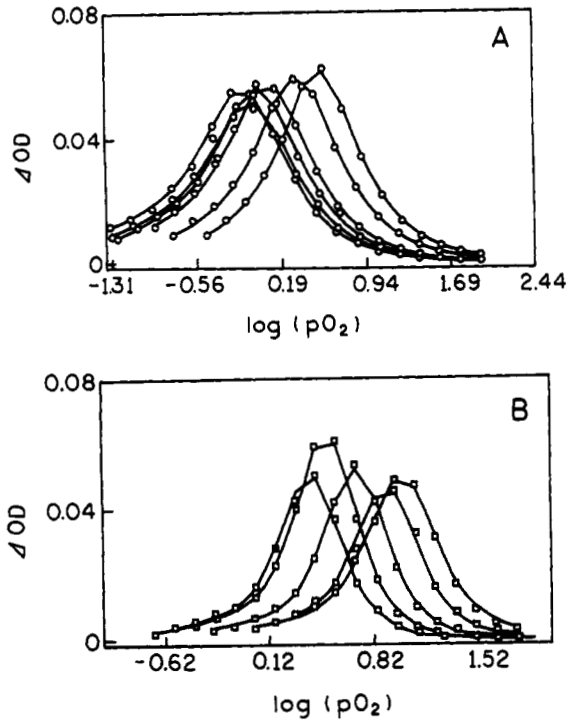

FIGURE 2: (A) Temperature-dependent oxygen binding isotherms of HbAFBDA in 0.1 M borate buffer at pH 9.0. Protein concentration was near 1.5 mM/heme. The temperatures were 22.0, 23.6, 25.1, 27.2, 33.2, and 37.7 °C, respectively. (B) Temperature-dependent oxygen binding isotherms of HbBAαXL in 0.1 M borate buffer at pH 9.0. Protein concentration was near 1.5 mM/heme. The temperatures were 22.4, 26.6, 29.8, 33.1, and 35.6 °C, respectively.

those analyses, we recovered the values of the β_i parameters shown in Tables II and III for HbAβXL and HbAαXL, respectively. In all curves, the maximum value of the Hill parameter was $n = 1.6 \pm 0.1$ for HbAβXL and 2.2 ± 0.1 for HbAαXL, respectively. The tables show the complete sets of data obtained either from local analyses of single isotherms or from the simultaneous (global) analyses of all the isotherms. It can be noted that the global analyses produced a progressive shrinking of the standard deviations of the estimated parameters with every additional isotherm. The resulting standard deviations were below 1% of the estimated values.

The thermodynamic quantities shown in Tables IV and V for the two cross-linked hemoglobins, respectively, were obtained from the data of global analyses using eq 4 for the intrinsic affinity constants and eq 6 for the $\Delta H°$ values of the individual steps of oxygenation.

The changes of the distribution of intermediates as function of the oxygen pressure are reported in Figure 3, where they are compared to the normal protein. The presence of the cross-link between the β-subunits increased the relative amounts of all the intermediates of oxygenation. For HbAαXL, the monoligated species increased, while the triligated species decreased to hardly detectable values.

The enthalpies of each oxygenation step of the two kinds of cross-linked hemoglobins are illustrated in Figure 4 where they are compared with those of normal hemoglobin. The anomalous endothermic behavior of step 3 in normal hemoglobin is even enhanced in HbAαXL, while the enthalpies in HbAβXL at each oxygenation step become similar. We attempted to make global analyses in which the enthalpies of each step of oxygenation were kept constant at the average values listed in Table IV and V for HbAβXL and HbAαXL, respectively. This produced simulations with very similar statistics for HbAβXL, confirming that the enthalpies of the various steps of oxygenation were very similar. This was not the case for the HbAαXL, where constant enthalpies values increased about 100-fold the standard deviations of the estimated parameters.

Figure 5 shows the van't Hoff plots of the P_m values obtained from local analyses superimposed to the lines described by the data of the global analyses, for the two cross-linked hemoglobins. The average enthalpies so obtained were identical to the average values listed in Tables IV and V with values of −19 and −12 kcal/heme for HbAβXL and HbAαXL, respectively. These values must be compared to the value −15 kcal/heme previously reported for HbA under the same conditions (Bucci et al., 1991). Again, these observations show that the two cross-links had a very different effect on the hemoglobin system.

DISCUSSION

Global analysis of the temperature-dependent isotherms at alkaline pH appears to be a powerful tool for investigating the conformation of the intermediates of ligation in hemoglobin. At alkaline pH, the hemoglobin molecule is "naked" in the sense that its interaction with anions and polyanions is practically eliminated. This eliminates the heats of solvent interaction and allows comparison of different systems like cross-linked and non-cross-linked species, and human and bovine hemoglobins, which interact differently with anions and have different Bohr effects. The technique acquires a greater validity from the observation that, both in this and our previous study (Bucci et al., 1991), the standard deviations of the estimated parameters progressively decreased with each additional isotherm added to the analyses. This also implies that the standard enthalpies of oxygen binding at each oxygenation step were identical at all temperatures, otherwise the opposite would have occurred to the point of preventing convergence of the floating parameters.

The different experimental conditions and especially the absence of interaction with anions prevents a detailed comparison of our results with those obtained in other laboratories on cross-linked hemoglobins. Nevertheless, there is a general agreement with the data of Miura et al. (1988) on β-cross-linked hemoglobin hybrids and with the data of Vandegriff et al. [1989] on α-cross-linked hemoglobin. It should be stressed that also Vadegriff et al. (1989) find that the overall constant β_3 acquires very low values at neutral pH.

Effect of the Cross-Links on the Enthalpy of the Subsequent Steps of Oxygenation. As we expected, the cross-link between the β-subunits produced similar enthalpy at each step of oxygenation. This, however was not the case for HbAαXL, where the constraints increased the anomalous endothermic characteristic of the third step of oxygenation noted for HbA (Bucci et al., 1991). Regarding the discontinuity of the enthalpies of the subsequent steps of oxygenation, Table VI shows the excess enthalpy at each step of oxygenation for cross-linked and natural human and bovine hemoglobins assuming that the enthalpy of the reaction of oxygen with the heme per se is −14 kcal/heme. The sum of their absolute values can be taken as a measure of the discontinuity of the enthalpies of the system. It clearly shows that, compared to that of the normal system, the variability decreased in HbAβXL while it increased in HbAαXL.

The overall enthalpy of the cross-linked species, as estimated from the P_m values, was about 4 kcal/heme more exothermic than that of the parent molecule in HbAβXL, while it was 3 kcal/heme less exothermic in HbAαXL. This confirms a net loss of endothermic components in HbAβXL as opposed to

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

	temp (°C) and mode												
	22.0		23.6		25.1		27.2		33.2		37.7		25.0
	L	G[b]	L	G[b]	L	G[b]	L	G[b]	L	G[b]	L	G[b]	G[b]
β_1 (torr^{-1}) SD	11.4 ± 8%	10.7	7.6 ± 9%	8.3	6.9 ± 15%	6.6	3.92 ± 6%	4.8	2.1 ± 6%	1.95	1.06 ± 11%	1.02	6.7
β_2 (torr^{-2}) SD	14.2 ± 7%	14.7	10.2 ± 8%	9.2	4.6 ± 14%	5.9	3.3 ± 4%	3.2	0.67 ± 7%	0.59	0.15 ± 11%	0.18	6.1
β_3 (torr^{-3}) SD	23.3 ± 7%	23	12.6 ± 11%	12.2	6.6 ± 15%	6.8	2.6 ± 5%	3.04	0.37 ± 7%	0.32	0.075 ± 12%	0.63	7.1
β_4 (torr^{-4}) SD	20.8 ± 8%	20.7	9.0 ± 10%	9.2	3.6 ± 13%	4.4	1.4 ± 3%	1.5	0.087 ± 7%	0.083	0.0096 ± 12%	0.1	4.4

[a] The overall constants are in torr^{-i}; the data at the reference temperature (25 °C) are from global analyses only. The standard deviations are given in percent of the estimated values (±percent). [b] Standard deviation <1% of the estimate value.

Table III: Local (L) and Global (G) Overall Adair Constants Describing the Oxygen Binding Isotherms of HbAαXL in 0.1 M Borate Buffer at pH 9.0[a]

	temp (°C) and mode										
	22.4		26.6		29.85		33.1		35.65		25.0
	L	G[b]	L	G[b]	L	G[b]	L	G[b]	L	G[b]	G[b]
β_1 (torr^{-1}) SD	2.61 ± 22%	2.43	1.6 ± 14%	1.6	1.15 ± 13%	1.16	0.85 ± 17%	0.85	0.62 ± 17%	0.67	1.87
β_2 (torr^{-2}) SD	1.03 ± 21%	1.07	0.54 ± 17%	4.9	0.24 ± 17%	0.27	0.14 ± 16%	0.15	0.117 ± 16%	0.096	0.67
β_3 (torr^{-3}) SD	0.16 ± 32%	0.118	0.075 ± 29%	0.59	0.036 ± 24%	0.035	0.02 ± 22%	0.021	0.013 ± 23%	0.014	0.077
β_4 (torr^{-4}) SD	0.16 ± 19%	0.166	0.046 ± 16%	0.039	0.012 ± 22%	0.013	0.004 ± 17%	0.005	0.002 ± 17%	0.002	0.068

[a] The overall constants are in torr^{-i}; the data at the reference temperature (25 °C) are from global analyses only. The standard deviations are given in percent of the estimated values (±percent). [b] Standard Deviation <1% of the estimated values.

Table IV: Thermodynamic Parameters Computed from the Adair Constants Shown in Table II for HbAβXL Hemoglobin[a]

	step 1	step 2	step 3	step 4	average
K (mM^{-1})	1008.1	394.2	1134.0	1610.2	
ΔH (kcal/heme)	−24.2	−21.0	−12.2	−18.8	−19.7
ΔG (kcal/heme)	−8.2	−7.6	−8.2	−8.4	−8.1
ΔS (cal/deg/heme)	−53.7	−45.0	−13.3	−34.8	−36.7
$T\delta S$ (kcal/heme)	−16.0	−13.4	−4.0	−10.3	−10.9

[a] We used the parameters recovered by global analyses at 25 °C. The standard deviations were all within ±10% of the estimated values.

Table V: Thermodynamic Parameters Computed from the Adair Constants Shown in Table III for HbAαXL Hemoglobin[a]

	step 1	step 2	step 3	step 4	average
K (mM^{-1})	303.6	155.1	111.9	2294.6	
ΔH (kcal/heme)	−11.4	−14.1	+34.5	−60.5	−12.9
ΔG (kcal/heme)	−7.4	−7.6	−6.8	−8.6	−7.5
ΔS (cal/deg/heme)	−13.2	−23.6	+138.8	−174.0	−18.03
$T\delta S$ (kcal/heme)	−13.9	−7.0	+41.3	−51.0	−5.4

[a] We used the parameters recovered by global analyses at 25 °C. The standard deviations were all within ±10% of the estimated values.

a net gain of endothermic components in HbAαXL. Figure 4 shows that this loss in HbAβXL and gain in HbAαXL were mostly due to the enthalpy of the third oxygenation step.

Involvement of the Quaternary Structure. It should be stressed that all of the excess enthalpies listed in Table VI involve a substantial amount of heat. The endothermic enthalpy of step 3 in HbA and HbAαXL cannot be explained on the basis of a progressive rupture, and formation of local hydrophobic and electrostatic interactions which, having enthalpies of opposite sign, would have a compensatory effect. Even a simultaneous rupture of all of the salt bridges described by Perutz (1990), when the third oxygen enters the molecule, would be without effect at pH 9.0 where there is no Bohr effect and most of the salt bridges are already broken. More likely, we are in the presence of hydrophobic modifications of large surface areas, due to rearrangements of the quaternary structure of the molecule.

The proposition of hydrophobic modifications of the surface of hemoglobin with oxygenation is supported by the observation

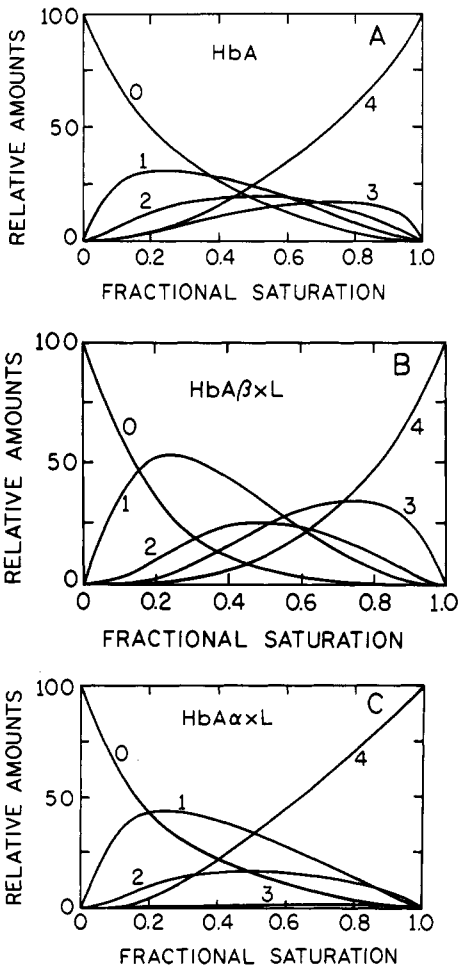

FIGURE 3: Dependence of the relative amounts of the intermediates of oxygenation on the fractal saturation with oxygen at 25 °C in (A) HbA, (B) HbAFBDA, and (C) HbAαXL. The data for HbA were taken from a previous publication (Bucci et al., 1991).

of Chotia (1974) that liganded hemoglobin has in excess of 900 Å^2 of hydrophobic surface more than the unliganded form. It is also consistent with the findings of Colombo et al. (1992)

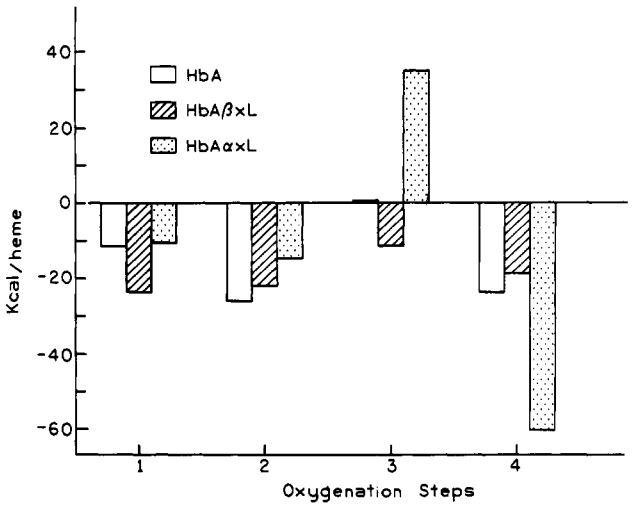

FIGURE 4: Enthalpy at subsequent steps of oxygenation of HbA, HbAβXL, and HbAαXL. The data for natural hemoglobin were taken from Bucci et al. (1991).

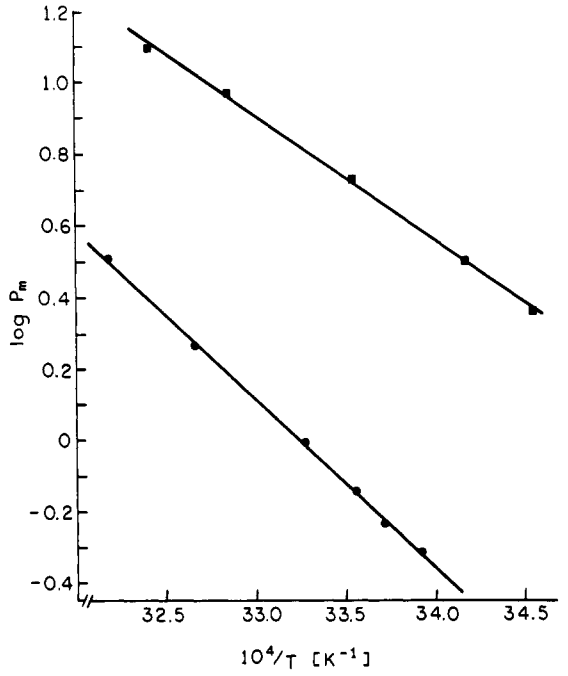

FIGURE 5: Van't Hoff plot of the temperature dependence of the P_m values of HbAβXL and HbAαXL. The overall enthalpy of oxygenation is estimated from the slope of the lines, $\Delta H^\circ = -19$ kcal/heme for HbAβXL and $\Delta H^\circ = -12$ kcal/heme for HbAαXL. The symbols are the experimental points, from local analyses. The interpolating lines are from global analyses.

Table VI: Excess Enthalpy, $\delta\Delta H$, of the Subsequent Steps, i, of Oxygenation of Cross-Linked and Natural Hemoglobins after Subtraction of the Enthalpy of the Oxygen–Heme Reaction, Which Is Assumed Constant at −14 kcal/heme

protein	$\delta\Delta H_1$ (kcal/heme)	$\delta\Delta H_2$ (kcal/heme)	$\delta\Delta H_3$ (kcal/heme)	$\delta\Delta H_4$ (kcal/heme)	$\sum\lvert\alpha\Delta H_i\rvert$ (kcal/heme)[a]
HbA	−1.4	−13.7	+14.5	−8.9	38.5
HbAβXL	−10.2	−7.0	+2.2	−4.9	24.3
HbAαXL	−2.6	0.0	+48.5	−46.5	97.6

[a] Sum of the absolute values.

that oxyhemoglobin binds 60 molecules of water per tetramer more than deoxyhemoglobin.

Thus, it appears that the different enthalpies of the third step of oxygenation in HbAβXL and HbAαXL are due to a different quaternary structure of the intermediate species.

Table VII: Distances between α_1–$\alpha_2$99 and β_1–$\beta_2$82 Lysine Pairs, Respectively, in Liganded and Unliganded Human Hemoglobin A and in Liganded Hemoglobin Ypsilanti

		liganded (Å)	unliganded (Å)
Hb	α99 pair	5.2	7.4
	β82 pair	10.7	9.3
Hb Ypsilanti	α99 pair	5.2	
	β82 pair	16.2	

Speculations on Novel Hemoglobin Structures. Smith et al. (1991) have demonstrated the existence in the hemoglobin system of a novel structure, the Y conformation, which presents an extensive rearrangement of the quaternary structure of hemoglobin. Peculiar to this conformation is the distance between the β-subunits, which exceeds that in either the R or the T conformations. As shown in Table VII, the distance between the β82 lysines goes from approximately 9 Å in the R and T structure to more than 16 Å in the Y structure. Instead, the reciprocal position of the α-subunits is much less modified, and the distance between the α99 residues in hemoglobin Ypsilanti remains that present in normal oxyhemoglobin.

The cross-link of fumaric acid spans about 8 Å and makes the opposite thermodynamic effects in HbAβXL and HbAαXL consistent with a Y-like structure acquired by the system upon binding of the third ligand. The quaternary structure rearrangement produced by the widening gap between the β-subunits in the Y-like structure would be responsible for the endothermic enthalpy noted in HbAαXL and in normal human hemoglobin (Bucci et al., 1991). In HbAβXL, the presence of the cross-link would prevent the formation of the Y-like structure and the appearance of endothermic enthalpy at the third step of oxygenation. In HbAαXL the widening gap between the β-subunits is not prevented, thereby sustaining and even emphasizing the endothermic enthalpy of the third oxygenation step.

These speculations give only a structural interpretation to the observation that, in normal hemoglobin and in HbAαXL, the transition between diligated and triligated species is accompanied by disappearance of hydrophobic surfaces, thereby liberating immobilized water molecules from the hydration shell, explaining endothermic effects. These surfaces are then reexposed to the solvent when the fourth oxygen binding produces the final R conformation, and water molecules are again immobilized, thereby explaining large exothermic enthalpys. This emphasizes the role of water in the allosteric transition of hemoglobin as proposed by Colombo et al. (1992).

In normal human hemoglobin, under certain conditions and especially at neutral pH and in the presence of chlorides (Bucci et al., unpublished results; DiCera et al., 1987; Gill et al., 1987; Vandegriff et al., 1989), the triligated species is very difficult to detect. Also, as already mentioned, the third step of oxygenation of normal hemoglobin is much more endothermic than the others (Bucci et al., 1991). This behavior is similar to that of HbAαXL and suggests that the cross-link between the α-subunits emphasizes an allosteric behavior normally present in the hemoglobin system.

[2] Note added in proof. A recent paper of Silva et al. (1992) came to our attention. The authors describe the structure of the R2-state of HbA, which has a quaternary structure very similar to that of hemoglobin Ypsilanti. This confirms that normal HbA has access to Y-like conformations.

Conclusion. These enthalpy data and those previously obtained (Bucci, et al., 1992) suggest that the oxygenation intermediates in hemoglobin acquire novel conformational features involving rearrangements of the quaternary structure. These rearrangements are especially evident for the third oxygenation step which produces a conformation where the β-subunits are probably further apart than in either the R or the T structures of the hemoglobin system, like in the Y structure of hemoglobin Ypsilanti (Smith et al., 1991). This model explains why the rearrangements of the quaternary structure are inhibited or even prevented by a cross-link between the β82 lysines produced by a fumaryl residue. Instead, the same cross-link between the α99 lysines does not prevent and possibly factors the formation of a Y-like structure at the third oxygenation step.[2]

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