Isolation and Characterization of the Triply Oxidized Derivative of a Cross-Linked Hemoglobin†

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ABSTRACT: Hemoglobin A, cross-linked between Lys 99α_1 and Lys 99α_2, was used to obtain a partially oxidized tetramer in which only one of the four hemes remains reduced. Because of the absence of dimerization, asymmetric, partially oxidized derivatives are stable. This is evidenced by the fact that eight of the ten possible oxidation states could be resolved by analytical isoelectric focusing. A triply oxidized hemoglobin population HbXL+3 was isolated whose predominant component was ($\alpha^+\alpha^+,\beta^+\beta^0$). This triferric preparation was examined as a possible model for the triliganded state of ferrous HbA. The aquomet and cyanomet derivatives were characterized by their CD spectra and their kinetic reactions with carbon monoxide. CD spectra in the region of 287 nm showed no apparent change in quaternary structure upon binding ligand to the fourth, ferrous heme. The spectra of the oxy and deoxy forms of the cyanomet and aquomet derivatives of HbXL+3 differed insignificantly and were characteristic of the normal liganded state. Upon addition of inositol hexaphosphate (IHP), both the oxy and deoxy derivatives of the high-spin triaquomet species converted to the native deoxy conformation. In contrast, IHP had no such effect on the conformation of the low-spin cyanomet derivatives of HbXL+3. The kinetics of CO combination as measured by stopped-flow and flash photolysis techniques present a more complex picture. In the presence of IHP the triaquomet derivative does bind CO with rate constants indicative of the T state whether these are measured by the stopped-flow technique or by flash photolysis. However, CO binding characterized entirely by R-state rate constants is observed only with the tricyanomet derivative in the absence of IHP and only when examined by flash photolysis. Under all other conditions, complex kinetics are observed which suggest the presence of more than one functional state of the protein.

The binding of oxygen to hemoglobin is a highly cooperative process. As a direct result of this, the fully deoxygenated and the fully liganded hemoglobin molecules predominate at equilibrium and the intermediate species with 1, 2, or 3 molecules of oxygen bound are present at far below their statistically predicted levels for a noncooperative system. Mills et al. (1976) have reported estimates of the concentrations of the intermediates as a function of oxygen saturation. Knowledge of the structures and functional properties of these species is essential to a detailed understanding of the linkage between ligand binding and the accompanying changes in protein structure [for review, see Perutz et al. (1987)].

Properties of these intermediates have been estimated from careful measurements of the bulk equilibrium properties and curve-fitting procedures [see, for example, the work of Mills et al. (1976) and Gill et al. (1987)] and from kinetic transients using such techniques as partial flash photolysis (Gibson, 1959). Obviously, it would be advantageous to be able to isolate these intermediates as pure components in solution. Two problems limit the feasibility of this approach. Ferrous ligands dissociate and recombine, rapidly causing them to reequilibrate with the available heme groups in times which are too short for the isolation and characterization of specific intermediates. Second, liganded hemoglobin exhibits a rapid equilibrium between the tetramer and $\alpha\beta$ dimer. As a result, even if ligand reequilibration were prevented, the only stable intermediates which could be produced would be those which are symmetric, i.e., with ligands on both α chains or on both β chains.

One solution to the first problem has been the use of partially oxidized hemoglobins as models of partially liganded intermediates. The rates of both intra- and intermolecular electron transfer between the heme groups in valency hybrids, in the absence of a redox mediator, are extremely slow, allowing the partially oxidized species to be isolated. Oxidation of Hb[1] shares many features with oxygen binding. During the oxidation of deoxyHb, the normal conformational transition occurs. Although the Hill coefficient is somewhat reduced, oxidation is a highly cooperative process (Bull et al., 1975). Furthermore, the quaternary structure of fully oxidized metHb is isomorphous with the fully liganded molecule (Heidner et al., 1976). Another approach is the use of mixed metal hybrids [see, for example, Blough et al. (1984)].

Both models have been used to study the symmetric intermediate species in which both α or both β chains are in the liganded state (Blough et al., 1984; Simolo et al., 1986; Mawatori et al., 1987; Luisi & Shibayama, 1989; Ishimori et al., 1989). However, disproportionation of the tetramer prevents the asymmetric species such as ($\alpha^+\alpha^0,\beta^0\beta^0$) or ($\alpha^+\alpha^+,\beta^+\beta^0$) from being isolated. To obtain asymmetric species, one must solve the second problem, i.e., prevent tetramer dissociation into dimers. Asymmetric valency hybrids of trout Hb have

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[1] Abbreviations: Hb, hemoglobin; HbA, human adult hemoglobin; metHb, methemoglobin; HbXL99α, hemoglobin cross-linked between Lys 99α_1 and Lys 99α_2; HbXL+3, triply oxidized cross-linked hemoglobin; IHP, inositol hexaphosphate; CD, circular dichroism; NMR, nuclear magnetic resonance; bis-Tris,[bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane]; Tris, tris(hydroxymethyl)aminomethane.

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been previously studied since the tetramer is unusually stable and does not undergo dimerization (Brunori et al., 1974). Alternatively, dimerization can be blocked by cross-linking the tetramer. Previously, we have described two cross-linked hemoglobins very well suited for this purpose, one cross-linked with bis(3,5-dibromosalicyl) fumarate between the two Lys 82β residues (Walder et al., 1980) and the other in which the α chains are cross-linked with the same reagent between the two Lys 99α residues (Chatterjee et al., 1986; Snyder et al., 1987). The α99 Lys cross-linking is achieved by reacting deoxygenated hemoglobin in the presence of IHP, this blocking the β82 sites with the organic phosphate and making available the alternate, α-chain sites. Both cross-linked hemoglobins remain highly cooperative. Asymmetric cyanomet valency hybrids of mixed tetramers composed of one HbA and one HbC dimer cross-linked as described were prepared by Miura et al. (1982, 1987). These were prepared by permitting symmetric forms of HbA and HbC (fully ferrous CO, fully cyanomet or symmetric dicyanomet diferrous CO) to equilibrate in solution so that their dimers associated randomly. After cross-linking, the asymmetric valency hybrids could be isolated as the HbA–HbC hybrids by their unique charge. Since the molecules being cross-linked were saturated with ligand, cross-linking was limited to the β82 position.

In going from the oxygenated to the deoxygenated states of HbA there is considerable rearrangement of the pocket between the two β chains while the motion of the α chains with respect to one another is quite small. It is therefore not surprising that the cross-link between the lysine 82 residues of the β-chains creates a greater distortion of the structure of deoxyhemoglobin than does that between the lysine 99 residues of the two α chains (Chatterjee et al., 1982, 1986). In addition, the β82 lysine cross-link blocks the organic phosphate binding site. For this reason the α99 cross-linked material would seem to be the better model for native HbA, although Larsen et al. (1990) have shown that even this cross-link produces some perturbation of the ligand-linked changes in the tertiary structure of the protein. Since this derivative forms only with the unliganded molecule, mixed valency hybrids must be prepared and isolated after cross-linking. We have undertaken the preparation and isolation of hybrids containing liganded ferrous and aquomet hemes. Isolation is based upon the difference in the charge of these species. In the present study, we focus on the triply liganded species. By characterizing the CD spectra and CO combination kinetics of various derivatives of HBXL+3, we explore the extent to which these derivatives resemble the deoxygenated, T state, the oxygenated, R state, or other unique structural states of the hemoglobin molecule.

Experimental Procedures

Materials. HbA was isolated from fresh whole blood as previously described and stored as a 15% solution frozen in liquid nitrogen (Walder et al., 1980). The cross-linked derivative, HbXL99α, was prepared by the reaction of deoxy-HbA with bis(3,5-dibromosalicyl) fumarate as described earlier (Chatterjee et al., 1986). Ampholines for isoelectric focusing were purchased from Pharmacia-LKB. IHP was obtained from Sigma as the sodium salt.

Analytical Isoelectric Focusing. Partially oxidized Hb species were analyzed by isoelectric focusing, using a modification of Perella's method (Perella et al., 1981, 1983). Gels were prepared using 2% (w/v) ampholines, pH 6.7–7.7 (Pharmacia-LKB)/20% w/v glycerol/5% acrylamide (acrylamide:bis 25:1). The catholyte solution was an 0.8% (w/v) mixture of ampholines (pH 7–9 and 8–9.5 in a 1:1 ratio). The anolyte solution was a 0.23% (w/v) solution of pH 6–8 ampholines dissolved in a cryosolvent containing 20% (v/v) ethylene glycol, 15% (v/v) MeOH, and 65% H_2O. The gels were focused at −5 °C for 5–8 h at 500 V and then fixed in 10% trichloroacetic acid. For quantitative analysis the gels were directly scanned with a 78/99 photodiode array digital camera (Eikonix Corp., Bedford, MA) and displayed using an IP8500 Image Processing System (Vicom Inc. Image and Graphics Division, Fremont, CA). The software package LONISP (Laboratory of Neuro-Imaging, Washington University, St. Louis, MO; Arthur Toga, Director) was used to control the digital camera and store the images.

Isolation of HbXL+3. HbXL+3 was prepared by oxidation of HbXL99α with ferricyanide (0.75 equiv/heme) in 100 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane(bis-Tris), pH 7.2, at room temperature for 15 min. The reaction proceeds to a level of nearly 75% metHb, which yields a maximal amount of the triply oxidized species. The fraction of metHb was determined spectrophotometrically from the absorbances at 577 and 630 nm. Any residual ferricyanide and generated ferrocyanide were removed by gel filtration using a Sephadex G-25 column. The triply oxidized product was isolated by preparative flat-bed isoelectric focusing at 4 °C using a LKB multiphor unit. Electrophoresis was carried out for 6 h at 700 V with a pH gradient of 6.7–7.7.

For most of the kinetic studies, HbXL+3 was prepared by an alternative technique. Purified HbXL99α was oxidized to approximately 75% metHb as already described. The mixture of the various oxidation states of HbXL99α was deionized by passage first through a Sephadex G-25 column equilibrated with 1 mM Tris-HCl, pH 8.4, and then through a Dintzis column (Riggs et al., 1981). This material was made 15 mM in Tris-HCl, pH 8.0, and then loaded onto a glass TSK gel DEAE-5 PW anion-exchange column (22 × 150 mm) on a Waters Model 650 preparative HPLC apparatus maintained at 10 °C. The column had been equilibrated with 15 mM Tris-HCl, pH 8, and elution was accomplished with a 425-mL linear gradient from the equilibration buffer to 15 mM bis-Tris-HCl, pH 7, at a flow rate of 5 mL/min. All buffers were equilibrated with air so that the ferrous heme groups were saturated with oxygen. Identification of the various fractions of metHb was determined spectrophotometrically from the absorbances at 540 and 500 nm. The second major peak was the triferric material. The central portion of this peak was harvested and rechromatographed as necessary. The purity of isolated material was assayed by analytical HPLC, and identification of the triply oxidized species was confirmed spectrally from the ratio of absorbances at 415 and 406 nm.

For kinetic comparisons native human HbA was prepared from freshly drawn blood according to the method of Geraci et al. (1969). After lysis, the ionic strength of the solution was raised to 0.1 M Cl^- with NaCl and then centrifuged at approximately 75000*g*. The hemolysate was passed through a Sephadex G-25 coarse column equilibrated with 1 mM Tris-HCl, pH 8.4. The eluate was passed through a Dintzis deionizing column of mixed-bed resin to remove ions, in particular, 2,3-disphosphoglycerate.

All kinetic measurements were carried out at pH 7 in bis-Tris buffer at a chloride concentration of 0.1 M. This buffer is prepared by titrating an amount of HCl equal to the desired chloride ion concentration with solid bis-Tris base to the desired pH and diluting appropriately. Inositol hexaphosphate, IHP (Sigma), was obtained in the sodium form. The pH of the stock solution (0.2 M) was adjusted to pH 5.6 with the protonated form of Amberlite IR-120 resin. IHP was always added to give a final concentration which was 1 mM in excess

of the concentration of hemoglobin tetramers.

Circular Dichroism Studies. CD spectra were recorded at 25 °C using an Aviv Associates CD spectrometer Model 60DS. A 20 μM Hb solution in 100 mM bis-Tris buffer, pH 7.2, was first deoxygenated under nitrogen in a quartz cuvette, shown to be deoxygenated by analysis of the visible spectrum, then transferred to an optical cell, and placed in the CD spectrometer. After the deoxy spectrum was recorded, the solutions were rapidly oxygenated and their CD spectra were again recorded. Ellipticity values are reported as molar ellipticity $[\theta]$ in units of deg cm^2 dmol^{-1}. Spectra were also obtained in the presence of IHP, at a concentration of 5–10-fold in excess over that of Hb.

Flash Photolysis. Carbon monoxide recombination following photodissociation was measured with a flash photolysis apparatus based upon the use of two photographic strobe lamps (Sunpak Auto 544) which are equipped with thyristor quenching devices. These strobe lamps were adjusted to give a rectangular light pulse approximately 0.6 ms in duration with rise and decay times < 0.1 ms. The flash was filtered with a solution of auramine in order to eliminate light at the wavelength of the observation beam. Reactions were followed at 420 and 435 nm. Data collection and processing was by a OLIS Model 4000 data acquisition and instrument control system. The sample was maintained at 20 °C. The concentration of hemoglobin was generally 5 μM in heme equivalents, i.e., 1.25 μM in ferrous heme for the triferric species. The CO concentration varied between 15 and 50 μM but was always 10-fold greater than that of the reactive ferrous hemes. To ensure anaerobic conditions, residual oxygen was removed from these solutions with an enzyme system consisting of 25 μg/mL beef liver catalase (Boehringer-Mannheim, 65 000 units/mg), 25 μg/mL glucose oxidase type VII (Sigma, 132 000 units/g), and 2.5 mM D(+)-glucose. With the use of this enzyme system it is essential that deoxygenation be complete before the addition of cyanide to the hemoglobin solution since this anion competitively inhibits the catalase. For the cyanomet triferric derivative, experiments were performed in the presence of 1 mM KCN which is sufficient to saturate the ferric hemes at pH 7.0. For these experiments, EDTA was added to a concentration of 10 μM.

Stopped Flow. The kinetics of combination of deoxygenated Hb with CO were also measured in a Dionex stopped-flow apparatus based on the Gibson-Durrum design (Gibson et al., 1964). The dead time of this instrument was estimated to be between 1.5 and 2.0 ms. To obtain anaerobic conditions, the stopped-flow apparatus was exposed to a deoxygenated buffer that contained dithionite. The system was washed thoroughly with large volumes of deoxygenated 1 mM Tris-HCl, pH 8.4. To test for any residual dithionite, ferric HbA was mixed with CO and the reaction, if any, was followed at 420 nm. Anaerobic conditions were maintained in the stopped-flow apparatus by inclusion of the catalase–glucose oxidase–glucose system in the hemoglobin and CO solutions. For most studies, a path length of 2 cm was used along with a hemoglobin concentration between 2.5 and 7.5 μM in heme after mixing. For a few studies on the triferric derivative, a 2-mm path length cuvette was used along with a Hb concentration after mixing of 22 μM (5.5 μM in reactive ferrous heme groups) and a CO concentration of 23 μM. Other conditions were as in the flash photolysis experiments.

Results

Separation and Assignment of Partially Oxidized Hb Species. Perella et al. (1981) have shown that separation of all valency hybrids of HbA can be accomplished by isoelectric focusing at −25 °C with the exception of $(\alpha_1^+\alpha_2^0,\beta_1^+\beta_2^0)$ and $(\alpha_1^+\alpha_2^0,\beta_1^0\beta_2^+)$ which comigrate. A variation of this procedure afforded good resolution of the partially oxidized intermediates of HbXL99α. Since the cross-link blocks dimer–tetramer exchange, it was not necessary to run the gel at extremely low temperatures to stabilize the asymmetric species. The valency hybrids were well resolved by isoelectric focusing at −5 °C (Figure 1). When the same system is employed at temperatures of 0 °C and higher, only the five macroscopic valence states ($Hb^0 \rightarrow Hb^{4+}$) are observed.

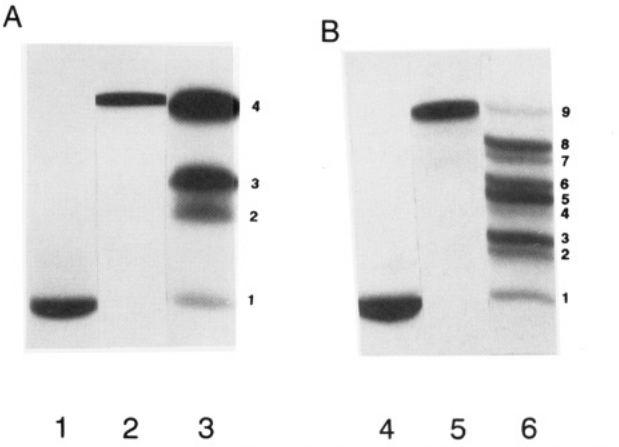

FIGURE 1: Isoelectric focusing of valency hybrids of HbA and HbXL99α. (A) Lanes 1 and 2 show HbA that is reduced and fully oxidized, respectively. Lane 3 is a partially oxidized HbA sample (60% met) containing the following symmetric species: 1, $(\alpha^0\alpha^0,\beta^0\beta^0)$; 2, $(\alpha^0\alpha^0,\beta^+\beta^+)$; 3, $(\alpha^+\alpha^+,\beta^0\beta^0)$; and 4, $(\alpha^+\alpha^+,\beta^+\beta^+)$. (B) Lanes 4 and 5 contain the cross-linked species, HbXL99α, that is reduced and fully oxidized, respectively. A partially oxidized sample of HbXL99α (50% met) is shown in lane 6. The assignments are as follows: 1, $(\alpha^0\alpha^0,\beta^0\beta^0)$; 2, $(\alpha^0\alpha^0,\beta^+\beta^0)$; 3, $(\alpha^+\alpha^0,\beta^0\beta^0)$; 4, $(\alpha^0\alpha^0,\beta^+\beta^+)$; 5, $(\alpha_1^+\alpha_2^0,\beta_1^+\beta_2^0)$ and $(\alpha_1^+\alpha_2^0,\beta_1^0\beta_2^+)$; 6,$(\alpha^+\alpha^+,\beta^0\beta^0)$; 7, $(\alpha^0\alpha^+,\beta^+\beta^+)$; 8, $(\alpha^+\alpha^+,\beta^+\beta^0)$; and 9, $(\alpha^+\alpha^+,\beta^+\beta^+)$. Hb species were oxidized with potassium ferricyanide in 100 mM bis-Tris buffer, pH 7.2. Isoelectric focusing was performed at −5 °C. The top of the gel is the cathodic end.

In Figure 1A, lanes 1–3 show native HbA oxidized to 0, 100, and 60% respectively. Only two intermediates are seen in the 60% oxidized sample. These have been identified previously as the symmetric doubly oxidized species $(\alpha^+\alpha^+,\beta^0\beta^0)$ and $(\alpha^0\alpha^0,\beta^+\beta^+)$ (Perella et al., 1981). Under the conditions of electrophoresis, the oxidized derivatives remain in the aquomet form. Oxidation of the α subunits leads to a greater increase in isoelectric point than that of their β counterparts (Park, 1973; Mast et al., 1976; Tomoda et al., 1978, 1979). Lanes 4–6 in Figure 1B show the isoelectric focusing pattern of HbXL99α at different levels of oxidation. Six of the eight intermediate valency hybrids are resolvable. As in the case of HbA, $(\alpha_1^+\alpha_2^0,\beta_1^+\beta_2^0)$ and $(\alpha_1^+\alpha_2^0,\beta_1^0\beta_2^+)$ have identical isoelectric points. The lower doublet, the intermediate triplet, and the upper doublet were each isolated by preparative isoelectric focusing and found to have the predicted levels of metHb: 25, 50, and 75%, respectively. At different levels of oxidation from 20 to 85%, the percent of metHb calculated from densitometric scans of the gel agreed within 3% of the value determined spectrophotometrically, further corroborating the assignments. The isoelectric point of HbXL99α, when in the liganded form, differs very little from that of HbA (Chatterjee et al., 1986). Correspondingly, the symmetric doubly oxidized species $(\alpha^+\alpha^+,\beta^0\beta^0)$ and $(\alpha^0\alpha^0,\beta^+\beta^+)$ nearly comigrate with their counterparts for native HbA. As with HbA, the α chains were preferentially oxidized.

Tomada and Yoneyama (1979) reported that the sensitivity of the β subunits to oxidation increases at high salt concentrations. We have used this observation to confirm the as-

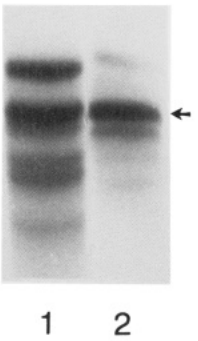

FIGURE 2: Isoelectric focusing of purified HbXL+3. Lane 1 shows HbXL99α partially oxidized to a level of 75% met using potassium ferricyanide under conditions described in Figure 1. The triply oxidized species isolated by preparative isoelectric focusing is shown in lane 2. The arrow indicates the predominant species, ($\alpha^+\alpha^+,\beta^+\beta^0$).

signment of the microscopic oxidation states of the cross-linked Hb. Addition of 250 mM NaCl during the oxidation with ferricyanide resulted in an increase in the intensity of the lower band at each of the three intermediate valency states such that nearly an equal ratio of α and β oxidized species was obtained (data not shown).

Isolation of HbXL+3. The first step in the preparation of the asymmetric, trivalent species, HbXL+3, was to partially oxidize HbXL99α with potassium ferricyanide to a level of 75% metHb. This yielded the maximal amount of the triply oxidized derivative (approximately 42% of the total population). The valency hybrids were separated by preparative isoelectric focusing in a pH 6.7–7.7 gradient at 4 °C. At this temperature, the macroscopic valency states 0, +1, +2, +3, and +4 are easily resolvable. The band migrating as the triferric (+3) state was isolated and characterized by analytical isoelectric focusing at −5 °C. Lane 2 in Figure 2 shows the isolated derivative where the predominant band, based on the previous assignments, is readily identified as ($\alpha^+\alpha^+,\beta^+\beta^0$). The ratio of the two bands corresponding to ($\alpha^+\alpha^+,\beta^+\beta^0$) and ($\alpha^+\alpha^0,\beta^+\beta^+$) was calculated by densitometric scanning to be 4:1. Trace amounts of the fully oxidized derivative are also present but would not interfere with the subsequent functional studies.

The triaquomet derivative was also isolated by preparative HPLC. The elution pattern of approximately 75% oxidized HbXL99α from the anion-exchange HPLC column is shown in Figure 3A. Absorbances at both 500 and 540 nm were monitored. The profile at 500 nm is shown in Figure 3A. The partially oxidized hemoglobins were eluted in order from most to least oxidized as expected from their relative net charges and as confirmed by the ratios of their absorbances at the two monitoring wavelengths. The elution pattern begins with a series of minor peaks in which the hemes are fully oxidized followed by a major band of ferric HbXL99α. The central portion of the second major peak, which corresponds to HbXL+3, was collected, and this material was rechromatographed until the diferric material represented no more than 3% of the total hemoglobin (Figure 3B). Fully ferric impurities (approximately 3%) could be tolerated because this material does not react in the kinetic studies reported here.

Circular Dichroism Studies of Hb Species. The CD spectra of HbXL+3 were used as a probe of its quaternary structure. The CD spectra for oxy- and deoxyHbA are shown in Figure 4A. Deoxygenation of HbA results in a prominent negative ellipticity in the region of 287 nm (Simon et al., 1969; Perutz et al., 1974a–c). The negative ellipticity arises from aromatic residues at the $\alpha_1\beta_2$ interface. Because of their location, these residues are sensitive to transitions of quaternary structure

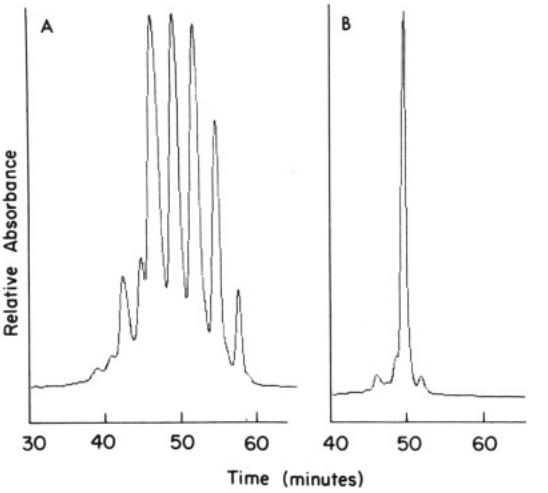

FIGURE 3: (A) Profile for the HPLC elution of 75% oxidized HbXL99α from a TSK gel DEAE-5PW anion-exchange column at 10 °C. The absorbance was monitored at 500 nm. A 5 mL/min, 85-min linear gradient from 15 mM Tris-HCl, pH 8 to 15 mM bis-Tris-HCl, pH 7, was used. The fourth peak from the right was identified as HbXL+3. (B) Analytical HPLC profile of purified HbXL+3 monitored at 406 nm. Experimental conditions were the same as for panel A.

from the R to the T state and not to details of ligand binding at the heme groups. The CD spectra for oxy- and deoxyHbXL99α are essentially identical to that of HbA, indicating that the presence of the cross-link does not substantially perturb the environment surrounding the aromatic residues at the $\alpha_1\beta_2$ interface (Figure 4B). As discussed above, this Hb has previously been shown to be highly cooperative and undergo the normal transition in quaternary structure upon ligand binding (Chatterjee et al., 1986; Snyder et al., 1987). Figure 4C shows the spectra for the triaquomet derivative HbXL+3. Upon deoxygenation of the remaining ferrous subunit, aquoHbXL+3 exhibits very little spectral change indicating that the tetramer remains globally in the liganded (R) conformation. To rule out the possibility that aquoHbXL+3 was locked in the R state, IHP was added. This brought about a change in the CD spectrum to that representative of a typical unliganded T-state Hb (see Figure 4C). The CD spectra for the low-spin tricyanomet derivative is shown in Figure 4D. Upon deoxygenation, the CD spectrum remains unchanged for cyanoHbXL+3, again indicating that the molecule remains in the R conformation. However, unlike aquoHbXL+3, the CD spectrum for cyanoHbXL+3 exhibits no change in response to addition of IHP.

Figure 5 summarizes the CD data by comparing the negative molar ellipticity values at 287 nm for all Hb species. The data can clearly be interpreted in terms of the existence of two quaternary states: those that are in the unliganded (T) conformation having negative molar ellipticity values near or above 100, and those species that are in the liganded conformation having values between 4 and 20. There appears to be no gross change in quaternary structure between deoxyHbXL+3 and the fully liganded molecule.

Kinetics of CO Binding. Preliminary to a study of the kinetics of CO binding to the triferric derivatives of HbXL99α, the kinetics of CO binding to the fully ferrous form of HbXL99α were compared to those of native HbA. The rates of CO combination to HbXL99α or HbA in the absence and presence of IHP at pH 6.9, 20 °C, were measured by both flash photolysis and by rapid mixing in the stopped-flow apparatus. The time courses of recombination of CO with HbXL99α and HbA following photodissociation are shown

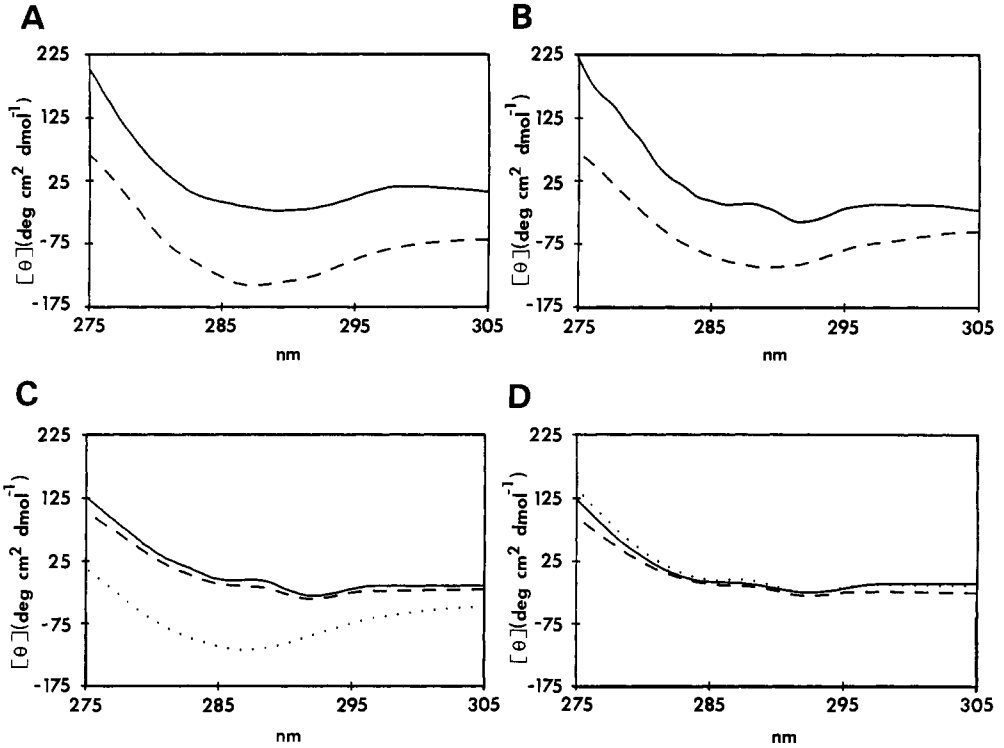

FIGURE 4: CD spectral studies of various hemoglobins at 25 °C in 100 mM bis-Tris/100 mM NaCl, pH 7.2. The Hb concentration of all samples is 20 μM tetramer. Panel A: oxyHbA (—) and deoxyHbA (---). Panel B: oxyHbXL99α (—) and deoxyHbXL99a (---). Panel C: oxyaquoHbXL+3 (—), deoxyaquoHbXL+3 (---), and deoxyaquoHbXL+3 with the addition of IHP (···). Panel D: oxycyanoHbXL+3 (—), deoxycyanoHbXL+3 (---), deoxycyanoHbXL+3 with the addition of IHP (···).

in Figure 6A. For HbA, the initial rate of the CO combination reaction is very fast and comprises 50% of the reaction. This fast phase is associated with the rate of CO combination to the large dimer population present in liganded HbA before photolysis and is represented by a rate constant of 5×10^6 M^{-1} s^{-1}. The presence of the cross-link between the α subunits in HbXL99α prevents dimer formation (Snyder et al., 1987). This is confirmed by the absence of a fast phase in the recombination kinetics.

As expected, the presence of IHP does not affect the dimer rate but does slow the reaction of the tetramer with ligand appreciably. The dimer–tetramer equilibrium of HbA is affected very little by the presence of 1 mM IHP. The stabilization of the tetrameric structure by IHP is well-known, but it occurs maximally at 0.1 mM IHP (White, 1976).

Because some of the studies on the triferric material were carried out in the presence of cyanide, the effects of this anion on the functional properties of the fully reduced, cross-linked hemoglobin were examined. Concentrations up to 30 mM cyanide had no effect on the observed kinetics of CO recombination (data not shown).

CO combination data for HbA and HbXL99α as measured by stopped flow are shown in Figure 6B. No fast phase was observed for either hemoglobin whether or not IHP was present. With stopped flow, the hemoglobin is deoxygenated before mixing with CO, and only the slower tetrameric rate of CO combination is expected and observed. The similarity of HbA and HbXL99α is apparent. In the presence of IHP, the kinetics of CO combination to these two hemoglobins are essentially identical. In the absence of IHP, HbXL99α initially binds CO somewhat more slowly than HbA, but at high fractional saturation the two hemoglobins exhibit the same final rate. Therefore, CO combination to HbXL99α is actually somewhat more autoaccelerating than its reaction with HbA.

With the availability of the stable triliganded Hb derivative, HbXL+3, it was possible to determine the rate of CO combination with the single unliganded ferrous heme and to determine how this rate was affected by organic phosphate and the nature of the ligands bound to the ferric hemes. The rates of CO combination to HbXL+3, in which either water or cyanide was bound to the ferric hemes, were measured by both stopped flow and flash photolysis. The time courses of these reactions in the absence and presence of IHP are shown in Figure 7. The calculated rate constants averaged from three or more experiments are presented in Tables I and II. Most

Table I: Rate Constants As Determined by Rapid Mixing for the Reaction of CO with HbXL+3: Effects of Different Ferric Ligands and IHP

		rapid process		slow process	
ferric ligand	IHP	rate constant ($\times 10^6$ M^{-1} s^{-1})	% of rxn[a]	rate constant ($\times 10^6$ M^{-1} s^{-1})	% of rxn[a]
H_2O	+	0.11 ± 0.02	50 ± 10	0.05 ± 0.01	50 ± 10
H_2O	-	1.0 ± 0.2	45 ± 4	0.11 ± 0.01	55 ± 4
CN^-	+	0.75 ● 0.05	65 ± 5	0.14 ± 0.02	35 ± 5
CN^-	-	4.0 ± 0.9	55 ± 15	0.68 ± 0.17	45 ± 15

[a] Percentage of the total absorbance change attributed to the process.

Table II: Rate Constants As Determined by Flash Photolysis for the Reaction of CO with HbXL+3: Effects of Different Ferric Ligands and IHP

		rapid process		slow process	
ferric ligand	IHP	rate constant ($\times 10^6$ M^{-1} s^{-1})	% of rxn[a]	rate constant ($\times 10^6$ M^{-1} s^{-1})	% of rxn[a]
H_2O	+	0.16 ± 0.05	15 ± 4	0.06 ± 0.01	85 ± 3
H_2O	-	1.5 ± 0.2	60 ± 3	0.16 ± 0.02	40 ± 4
CN^-	+	1.2 ± 0.2	65 ± 7	0.13 ± 0.02	35 ± 4
CN^-	-	7.5 ± 1.5	60 ± 9	2.0 ± 0.6	40 ± 4

[a] Percentage of the total absorbance change attributed to the process.

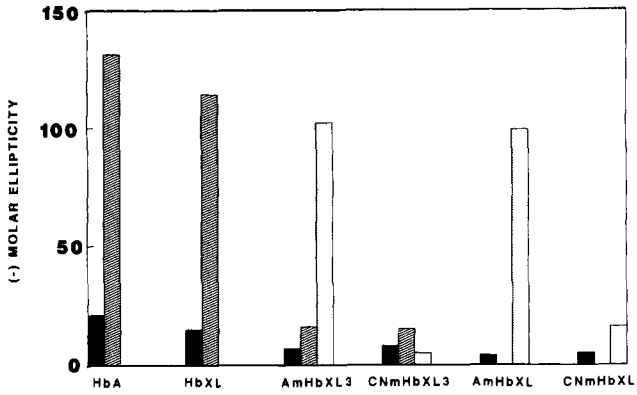

FIGURE 5: Summary of circular dichroism data. The bar graph represents the negative molar ellipticity values at 287 nm for (left to right) ferrous native HbA, ferrous-cross-linked HbA, triaquometHb, tricyanometHb, aquomet-cross-linked HbA, and cyanomet-cross-linked HbA: (black bars) oxy, (shaded bars) deoxy, and (white bars) deoxy + IHP. All molar ellipticity values are calculated from the molecular weight of Hb (64 500) and are expressed in units of deg cm^2 $dmol^{-1}$.

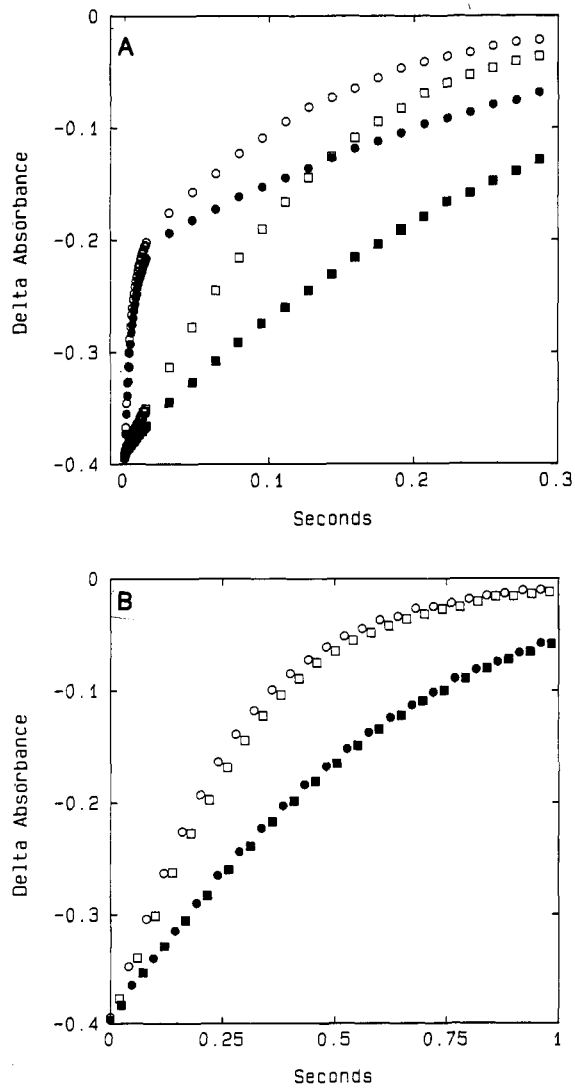

FIGURE 6: Time courses for the combination of carbon monoxide with native (circles) and cross-linked (squares) ferrous HbA at 20 °C in the absence (open symbols) and presence (closed symbols) of 1 mM IHP. Buffers were 0.1 M bis-Tris-HCl, pH 6.9. Conditions: (A) flash photolysis, [Hb] = 5 μM (heme) and [CO] = 50 μM; (B) stopped flow, [Hb] = 2.5 μM (heme) and [CO] = 25 μM.

of the data presented were collected on HbXL+3 isolated by HPLC. The kinetic traces shown in Figure 7 were obtained

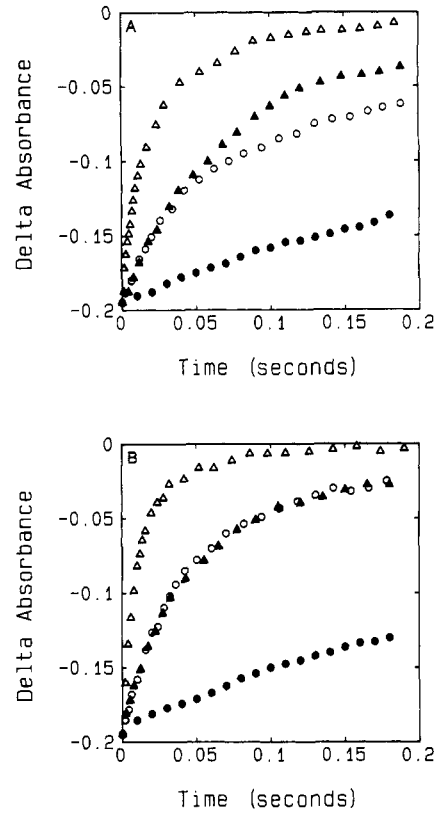

FIGURE 7: (A) Time courses for the combination of CO with the ferrous heme of HbXL+3 following rapid mixing in a stopped-flow instrument. The ferric ligands were either water (circles) or cyanide (triangles). Rates were measured at 420 nm in the absence (open symbols) and presence (closed symbols) of 1 mM IHP. Experiments were carried out at 20 °C in 0.1 M bis-Tris-HCl buffer, pH 6.9; [CO] = 24 μM; [HbXL+3] = 2.5 μM in ferrous heme; [CN^-] = 1 mM. The starting Δ absorbance has been normalized to a value of −0.195. (B) Time courses for the recombination of CO to HbXL+3 following flash photolysis. Symbols correspond to those in panel A. Conditions were the same as those in the stopped-flow experiments except that the reactive heme concentration was 1.25 μM.

using this material. However, some measurements were also carried out on hemoglobin purified by isoelectric focusing. No significant differences were detected. The rates of CO combination with the ferrous heme of HbXL+3 vary widely with experimental conditions, but under all conditions examined the kinetics are heterogeneous, being minimally biphasic, i.e., requiring at least two exponential functions in order to be fitted. In Tables I and II the second-order rate constants associated with each phase are reported along with the percentage of the total reaction which is represented by each phase.

CO combination measured by the stopped-flow technique is fundamentally different from CO recombination as measured by flash photolysis. In the former case, the starting material is the triliganded hemoglobin molecule with a single deoxygenated ferrous heme. In the latter case, one begins with a fully liganded molecule in which the ferrous heme is occupied by a carbon monoxide molecule. If these two procedures result in different kinetic behavior, the implication is that the structure of the fully liganded molecule differs in some regard from that of the triliganded molecule and the relaxation of the photolytically produced triliganded molecule to its equilibrium is slower than the rate of CO binding.

Considering first the data obtained by stopped-flow techniques (Table I), one observes that in the presence of IHP, when the ferric hemes are in the aquomet form, CO binds to the ferrous heme most slowly. There are two, roughly equal

kinetic phases with rate constants that differ by 2-fold. Both of these rate constants fall within the range expected for the T state of human Hb. The most rapid combination with CO is observed when the ferric hemes are bound to cyanide and when IHP is absent. Again, there are two kinetic phases, this time with rate constants that differ by 6-fold. The more rapid, 4×10^6 M^{-1} s^{-1} is typical of the rate of CO binding to the R state of human HbA while the slower corresponds to neither an R-state nor a typical T-state rate constant, being too slow for the former and much too fast for the latter. Intermediate kinetic behavior is observed with the triaquomet derivative in the absence of IHP and with the tricyanomet derivative in its presence (see Figure 7A). Though not identical, both of the reactions are composed of a slow phase with a rate constant corresponding to the T state and a faster phase with a rate constant somewhat too slow for the R state of HbA. Overall, the reaction of CO with the tricyanomet derivative in the presence of IHP appears to be the more rapid of the two. This is due almost entirely to its fast phase being a larger percentage of the total reaction.

When CO recombination to HbXL+3 following flash photolysis is examined under the same experimental conditions (Figure 7B), the pattern of dependence of the overall reaction on IHP and saturation with cyanide remains essentially the same. However, in this case the two intermediate time courses (tricyanomet plus IHP and aquomet without IHP) are much more similar to one another. As with the reactions observed with the stopped-flow apparatus, all of these time courses are heterogeneous and are fitted to the sum of two exponentials. The most striking difference observed is that recombination following flash photolysis is always more rapid than ligand binding after the mixing of reactants. This is clearly seen by comparing panels A and B in Figure 7. That the individual rate constants determined by flash photolysis are always greater than those measured by stopped flow is seen by comparing Tables I and II. There is also a large difference in the relative amounts of fast and slow phases for the reaction of CO with the triaquomet derivative in the presence of IHP, the ratio of the size of the fast to that of the slow phase being unity in the stopped flow and roughly 0.2 in flash photolysis.

During the flash photolysis studies we observed that the ferric hemes of aquomet HbXL+3 in the presence of carbon monoxide are reduced to the ferrous state, and this process is greatly accelerated upon exposure to light. Photoreduction could be detected by a progressive increase in the change in absorbance accompanying flash photolysis at constant flash energy and by a decrease in the ratio of absorbance at 406 and 420 nm. This is probably the same process as the CO reduction of Hb reported by Bickar et al. (1984), but these authors did not report an effect of light exposure. It may also be related to the transient photoreduction of aquomet Hb in the absence of CO reported by Kitagawa and Nagai (1979). Great care was required in the flash photolysis studies to avoid appreciable formation of other oxidation intermediates as a result of this reduction reaction. In spite of such precautions a third slow kinetic phase indicative of CO binding to a T state was observed in CO recombination to the tricyanomet derivative in the absence of IHP, the amount of which varied from zero to as much as 14% of the total reaction. Since its amount was so variable it was concluded that it did not represent a property of the triferric hemoglobin and might be due to some photoreduction. This suggestion was reinforced by our failure to observe this kinetic phase in stopped-flow experiments where photoreduction should not be a serious a problem because exposure of the hemoglobin to CO and light is very brief.

Discussion

With the availability of the cross-linked hemoglobin, HbXL99α, both symmetric and asymmetric partially oxidized species can be isolated. All of the microscopic states are resolvable by analytical isoelectric focusing except for ($\alpha_1^+\alpha_2^0,\beta_1^+\beta_2^0$) and ($\alpha_1^+\alpha_2^0,\beta_1^0\beta_2^+$) (see Figure 1B). On a preparative scale, only the five macroscopic states $Hb^0 \rightarrow Hb^{4+}$ can be separated by either isoelectric focusing or ion-exchange HPLC. Only two triferric species are possible, one in which an α heme remains reduced and one in which a β heme remains reduced. The triferric hemoglobin produced under our conditions for oxidation is a mixture of 20% of the former and 80% of the latter. Therefore, the properties we have measured reflect primarily those of the triferric molecule with a ferrous β subunit.

The properties of the triferric mixed valency hybrid are surprisingly complex. The CD results seem at first glance very straight forward, but there is limited correlation between the spectral properties and the functional properties of the heme groups as assayed by CO combination kinetics. Our CD results indicate that the deoxy forms of both cyano- and aquoHbXL+3 assume an R-state configuration. This conclusion is based on CD data taken in the near-ultraviolet region in which one observes the effects of changes in the environments of aromatic residues. These spectral changes, centered around 287 nm, have been shown to be characteristic of transitions in quaternary state and independent of the state of ligand saturation of the heme groups (Perutz et al., 1974a–c).

Spectra obtained in the presence of IHP clearly show that the cross-link in HbXL+3 does not aberrantly lock this molecule in the R state. Binding of IHP to aquometHbA, a high-spin derivative, has been reported to change the conformation of the tetramer from an R state to a T state and induce the characteristic negative molar ellipticity peak at 287 nm in the CD spectrum (Perutz et al., 1974a). Our results are the same with both oxy- and deoxyaquoHbXL+3, indicating that the tetramer is free to adopt the T quaternary structure. This is consistent with earlier oxygen binding studies of fully ferrous HbXL99α, which show essentially a normal level of cooperativity (Snyder et al., 1987), and with the crystal structure of its deoxygenated derivative (Chatterjee et al., 1986). IHP had no effect on the CD spectrum of cyanoHbXL+3, a low-spin derivative, as observed previously for cyanometHbA. The linkage between spin state and the quaternary structure equilibrium seen with Hb in the fully oxidized form is thus maintained when one of the subunits is in the deoxy state.

The kinetics of CO combination to HbXL+3 indicate a more complex situation than that revealed by CD spectroscopy. There is one situation, aquoHbXL+3 in the presence of IHP, in which the kinetic properties observed by both stopped flow and flash photolysis are those of a T state and one, cyanoHbXL+3 in the absence of IHP, in which kinetic properties measured by flash photolysis are consistent with an R state. Under these conditions, the kinetic and CD results are in agreement. The correspondence between these kinetic properties of tricyanoHbXL+3 and those of triliganded ferrous HbA suggest that the cyanomet derivative is the best model for the ferrous liganded heme. However, when one examines either cyanoHbXL+3 in the presence of IHP or aquoHbXL+3 in its absence by stopped flow or flash photolysis, one discovers a very interesting situation. Here, in spite of CD spectra which indicate that the protein is in the R state, we observe kinetic properties which suggest a mixture of functional states and

which are certainly not consistent with those classically assigned to the R state. In these instances, there are two distinct kinetic phases, the slower of which represents 35–55% of the total reaction and has a second-order rate constant between 1.1 and 1.6 X 10^5 M^{-1} s^{-1}, an order of magnitude too slow for the R state. Kinetic properties of cyanoHbXL+3 in the absence of IHP when measured by stopped-flow mixing are also not easily assigned to an R state. Here, there are again two kinetic phases, the slower of which has a rate constant that is roughly 3-fold too small for such an assignment.

Under every solvent condition examined, CO combination with HbXL+3 is heterogeneous, being minimally comprised of two second-order processes with different rate constants. One source of such heterogeneity is the α and β subunits. However, if in every case this were the only explanation, the relative amplitudes of the two phases should be constant, and they are not. Furthermore, these amplitudes should reflect the 80% ferrous β chain and 20% ferrous α chain ratio in our HbXL+3 preparations, and they clearly do not. This necessitates an explanation other than or in addition to subunit heterogeneity. One possible explanation is the occurrence of functionally distinct conformers which interconvert slowly compared to the time for CO combination. The occurrence of such slow conformational relaxations is clearly established by the observation that in general CO combination with HbXL+3 as measured in the stopped-flow apparatus is slower than the recombination of CO with HbXL+3 following flash photolysis. This means that the average rate at which ligand combines with the deoxy ferrous subunit of HbXL+3 depends in part on how long that subunit has been unliganded. Whether such slow relaxations are unique to HbXL+3 or are also a property of triliganded HbA is of course not known. They certainly do not result from an intrinsic limitation in the rate of the R to T state quaternary relaxation which is very rapid for HbA and appears to also be rapid for HbXL99α. CO combination to this cross-linked Hb following complete photodissociation is entirely slow, requiring that the R state to T state transition be complete before significant CO recombination from the solvent phase occurs.

It is generally accepted that triliganded ferrous HbA in the absence of organic phosphates exists in the R-state conformation. If one examines this assignment in terms of the functional properties of the remaining deoxy heme in this structure, one finds that it is based for the most part upon the fitting of ligand binding isotherms to the two-state model or estimates of kinetic properties by flash photolysis. As an estimate of the functional state of the triliganded molecule, the latter technique is subject to errors from slow relaxation as observed in the present work. Careful measurement of binding isotherms and the hemoglobin concentration dependence of such isotherms by Mills et al. (1976) and Gill et al. (1987) indicates that ligand binding to HbA cannot be precisely described by the two-state model. The assignment of the triliganded hemoglobin molecule to the R state has been challenged by the results obtained by the modulation excitation technique of Ferrone et al. (1985). These authors have concluded that under their experimental conditions 25% of triliganded HbA is in the T state. Our data suggest that under appropriate conditions the triferric model of triliganded hemoglobin can exist in the T state but that generally it exists in a state or states that are not easily described in the two-state formalism. These are states in which the CD spectrum indicates the R structure but in which kinetic properties of the single ferrous heme suggest something quite different. The variability of the kinetic properties is consistent with the existence of an equilibrium between multiple functional states, which might be related to the structural substates suggested by the work of Ansari et al. (1986). This equilibrium would have to be sensitive to the presence of ligand on the ferrous hemes, the nature of the ligand on the ferric hemes, and the presence of IHP and include processes which relax slowly with respect to the rate of CO combination.

Acknowledgments

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Registry No. CO, 630-08-0; IHP, 83-86-3.

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Contribution of Hydrogen Bonding to the Conformational Stability of Ribonuclease T1†

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ABSTRACT: For 30 years, the prevailing view has been that the hydrophobic effect contributes considerably more than hydrogen bonding to the conformational stability of globular proteins. The results and reasoning presented here suggest that hydrogen bonding and the hydrophobic effect make comparable contributions to the conformational stability of ribonuclease T1 (RNase T1). When RNase T1 folds, 86 intramolecular hydrogen bonds with an average length of 2.95 Å are formed. Twelve mutants of RNase T1 [Tyr → Phe (5), Ser → Ala (3), and Asn → Ala (4)] have been prepared that remove 17 of the hydrogen bonds with an average length of 2.93 Å. On the basis of urea and thermal unfolding studies of these mutants, the average decrease in conformational stability due to hydrogen bonding is 1.3 kcal/mol per hydrogen bond. This estimate is in good agreement with results from several related systems. Thus, we estimate that hydrogen bonding contributes about 110 kcal/mol to the conformational stability of RNase T1 and that this is comparable to the contribution of the hydrophobic effect. Accepting the idea that intramolecular hydrogen bonds contribute 1.3 ± 0.6 kcal/mol to the stability of systems in an aqueous environment makes it easier to understand the stability of the "molten globule" states of proteins, and the α-helical conformations of small peptides.

Most of the important tasks in living cells are carried out by proteins in which the polypeptide chain is tightly folded into a globular conformation that is essential for their biological activity. Consequently, there is great interest in the forces that stabilize globular proteins (Creighton, 1991; Dill, 1990). In early discussions of protein structure, hydrogen bonding was thought to be the most importance force contributing to the conformational stability. The main proponent of this view was Linus Pauling, who wrote with Mirsky (1936): "The importance of the hydrogen bond in protein structure can hardly be overemphasized." However, by the 1950's, the emphasis had shifted, and the importance of the hydrophobic effect was stressed first by Kauzmann (1959) and later by Tanford (1962), who used model compound data and calculations based on a simple model to conclude: "...the stability of the native conformation in water can be explained...entirely on the basis of the hydrophobic interactions of the non-polar parts of the molecule." The view that the hydrophobic effect makes a more important contribution than hydrogen bonding to globular protein stability is still widely held today. As recent examples, Kim and Baldwin (1990) state: "Stripping H_2O from nonpolar side chains to form a hydrophobic core provides the main source of free energy stabilizing a folded protein." Creighton (1990) states: "Nevertheless, the hydrophobic interaction is probably the major stabilizing factor." In a recent review that gives an excellent overview of the forces contributing to globular protein stability, Dill (1990) states: "More than 30 years after Kauzmann's insightful hypothesis, there is now strong accumulated evidence that hydrophobicity is the dominant force of protein folding, provided that "hydrophobic" is operationally defined in terms of the transfer of nonpolar amino acids from water into a medium that is nonpolar and

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