Characterization of Intermediate States in the Ligation of Hemoglobin†

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ABSTRACT: The binding of carbon monoxide to hemoglobin in the presence of diphosphoglyceric acid has been investigated by ^{19}F nuclear magnetic resonance spectroscopy. The ^{19}F nmr spectrum of hemoglobin trifluoroacetonylated at cysteine-β93 exhibits chemical shift changes on binding of ligands to the β chains. Comparison of these changes to the fractional ligation of all chains, determined concurrently from the fractional change in the visible spectrum, shows that ligands bind preferentially to α chains. The ^{19}F nmr spectrum of partially liganded hemoglobin (trifluoroacetonylated) contains resonances at the normal chemical shift positions of the fully liganded and deoxy species, in addition to two small resonances at intermediate positions. Analysis of the relative magnitudes of these four peaks as functions of carbon monoxide pressure permits identification of the intermediate species.

The problem of explaining the kinetics and equilibria of ligand binding to hemoglobin in molecular terms has been studied exhaustively from the standpoints of various models (Monod *et al.*, 1965; Koshland *et al.*, 1966; Hopfield *et al.*, 1971; Ogata and McConnell, 1972) and structural analysis (Perutz, 1970). Several models have been advanced which are consistent with the most precise binding data available, on the basis of entirely different (but kinetically indistinguishable) molecular interactions. Structural analysis of the end points of the allosteric transition was an important advance toward understanding of the molecular forces involved in stabilization of two forms of the protein, but the intermediate species generated during the binding process itself are not accessible to crystallographic analysis. Hence, precise knowledge of the deoxy- and met- or oxyhemoglobin structures has not resolved a number of mechanistic questions about the conversion process.

Some information on the intermediate stages of ligand binding has been obtained from spectroscopic studies in solution. The nuclear magnetic resonance (nmr) studies of Shulman and coworkers (Hopfield *et al.*, 1971) on "artificial" intermediates and the work of Ogata and McConnell (1972) on binding of allosteric effectors have yielded information on some of the changes in the molecule during processes approximating normal ligand binding. The model binding process which these workers propose concurs with the concerted model (Monod *et al.*, 1965) in most respects, but contains some aspects which resemble the sequential model (Koshland *et al.*, 1966).

The basic premise of all the models is that hemoglobin exhibits at least two forms which differ in structure and in ligand affinity. The differences between the models arise over the number of structures which exist, the role (if any) played by the ligand itself in the transition, and the relative energetic importance of various regions of the molecular structure to stability of the various forms.

The studies described in this communication yield information on some of these questions, using the nuclear magnetic resonance spectrum of trifluoroacetonylated hemoglobin (Hb^{TFA})[1] to monitor the equilibrium populations of the species present in the intermediate stages of ligation.

In Hb^{TFA}, the ^{19}F nmr probe situated at Cys-β93 undergoes observable environmental changes when hemoglobin binds ligands or undergoes structural changes induced by allosteric effectors. These changes directly reflect events in the β chain which reveal the presence of partially liganded species of intermediate structure. Comparison of changes in the Cys-β93-His-β146 region to fractional ligation of the entire tetramer (observed concurrently from changes in the visible spectrum) suggests a model for the sequence of ligand binding.

Experimental Section

Methods and Materials

Hemoglobin. Human hemoglobin was prepared from freshly drawn citrated blood. The packed erythrocytes were washed three times with 0.9% sodium chloride solution, and lysed with distilled water or distilled water and toluene. The stroma were removed by centrifugation and the supernatant was desalted by gel filtration through a Bio-Gel P-2 column (2.5 × 45 cm) equilibrated with a buffer containing 0.05 M bis-tris and 0.1 M NaCl at pH 7.0. Hemoglobin solutions were stored at 4° and used within four days of preparation. Trifluoroacetonylated hemoglobin was prepared as described previously (Huestis and Raftery, 1972a).

Reagents. Diphosphoglyceric acid was obtained from Calbiochem as the pentacyclohexylammonium salt and converted to the acid by being stirred with Dowex 50-X8. *p*-Hydroxymercuribenzoate was obtained from Sigma Chemical Co. Bromotrifluoroacetone was a product of Peninsular Chemresearch, Inc.

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[1] Abbreviations used are: Hb, hemoglobin; Hb^{TFA}, hemoglobin trifluoroacetonylated at Cys-β93; BuNC, *n*-butyl isocyanide; CO, carbon monoxide; DPG, 2,3-diphosphoglyceric acid; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrotriethanol; Y, the fraction of liganded hemoglobin = $[Hb\text{-}O_2]/[Hb] + [Hb\text{-}O_2]$; α,β, unliganded chains in low-affinity conformations; α^*,β^*, unliganded chains in high-affinity conformations; α^L,β^L, liganded chains; Y_β, the fraction of liganded β chains = $[\beta^L]/[\beta^L] + [\beta^*] + [\beta]$.

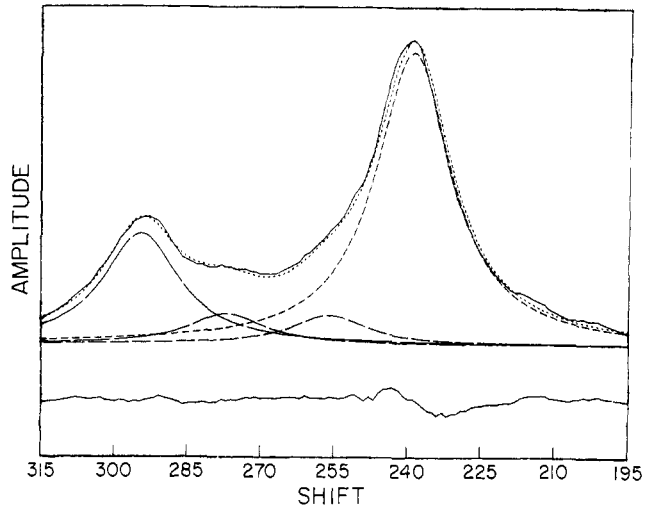

FIGURE 1: Calculated and observed nmr spectra of Hb^{TFA} when $Y = 0.40$. The solid line is the observed spectrum, the dotted line is the calculated sum of four Lorentzians, and the dashed lines are the individual Lorentzians. The error is plotted below the spectra.

Methods. Visible and nmr spectra were observed concurrently at various stages of carboxygenation using an nmr tube (12-mm o.d.) with a cuvet (1-mm optical path) fused to the top. This combination cell was made to order by Wilmad Glass Co. During an experiment, a small access hole sealed by a rubber septum permitted injection of gases into the tube by means of a syringe. Visible absorbances were determined with a Gilford Model 240 spectrophotometer. ^{19}F nmr spectra were recorded using a Varian XL-100 spectrometer with fluorine Fourier transform capability. The temperature of the nmr probe was 27°; the air temperature in the Gilford sample compartment was 26–27°. pH measurements were made using a Radiometer Copenhagen Model 26 pH meter.

Carbon Monoxide Binding Experiments. Nmr solutions contained 580 mg of Hb^{TFA} in 4.5 ml of bis-tris–NaCl buffer (pH 7.40 or 6.75, 0.05 M bis-tris–0.1 M NaCl). The large solution volume was necessary to prevent vortex formation when the tube was spun. A twofold molar excess of DPG was introduced as a concentrated solution of the appropriate pH. The hemoglobin solution was deoxygenated by repeated washing with nitrogen in a tonometer. The solution was transferred to the argon-filled sample tube by syringe, care being taken to avoid possible exposure to oxygen. Absorbance of the hemoglobin solution was determined at 540 nm before and after each nmr spectrum was recorded. Aliquots of carbon monoxide were injected into the tube and the tube was rotated manually to allow the solution to equilibrate with the gas mixture. When the absorbance had reached a constant value after each addition, the nmr spectrum was recorded. Owing to the small gas volume in the nmr tube, CO partial pressures were difficult to calculate accurately. The ordinate numbers in Figure 3 were determined from carbon monoxide binding curves obtained using dilute hemoglobin solutions.

Preparation of $\alpha_2^{IIICN}\beta_2$. The ligand state hybrid $\alpha_2^{IIICN}\beta_2$ was prepared as described by Ogawa and Shulman (1971), using isolated α and β chains prepared by the procedures of Kilmartin and Wootton (1970). The oxygenated hybrid was trifluoroacetonylated by the procedure used for Hb-A. Partial deoxygenation was achieved by washing the solution with nitrogen in a tonometer for 2 hr. The temperature of the solution was maintained below 10° except when nmr spectra were being recorded, to retard "heme exchange."

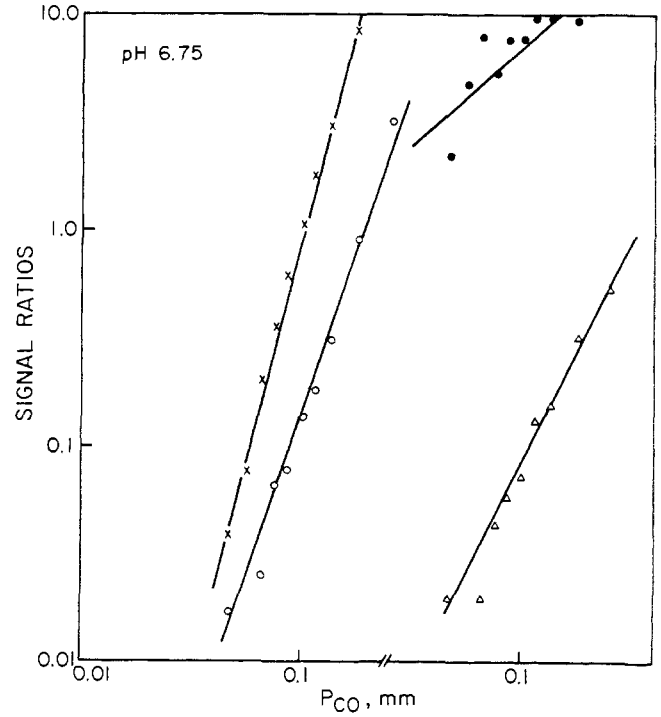

FIGURE 2: Dependence of the nmr populations on P_{CO} at pH 6.75 in the presence of DPG: (×) L/D; (○) I_3/E; (●) L/I_3; (▲) I_2/D. The slopes, determined by least-squares fits, are 4.08, 3.22, 2.06, and 0.9, respectively.

Analysis of Nmr Data. The positions and relative magnitudes of the resonances in nmr spectra were determined by use of a Lorentzian fitting program. Figure 1 shows the results of a typical fit, the four component peaks and the error being plotted below the calculated and observed spectra. The species contributing to each of the four resonances were identified from the slopes of log–log plots of peak ratios and carbon monoxide pressures (Figure 2).

Results

The ^{19}F nmr spectrum of Hb^{TFA} changed with increasing fractional ligation (Y) as is shown in Figure 3. In addition to the absorbances characteristic of deoxy- (D) and carboxyhemoglobin (L), two additional resonances were present in the intermediate ligation range. The larger of these resonances, I_3, appeared 15 cps upfield of the Hb^{TFA}-CO resonance (L) and, like L, its chemical shift position was independent of pH in the range 6.75–7.40. The smaller intermediate resonance (I_2) appeared approximately 20 cps downfield of the deoxyhemoglobin peak (D), but its position varied when DPG was added or the pH was changed. I_3 and I_2 contained about 8 and 4% of the total nmr integral, respectively. The positions of all four peaks were independent of the carbon monoxide pressure.

Nmr Studies of the Artificial Intermediate $\alpha_2^{IIICN}\beta_2$. Analysis of the ligand binding data in molecular terms was dependent on unequivocal identification of the subunits influencing the nmr probe. For this purpose, the ligand state hybrid $\alpha_2^{IIICN}\beta_2$ was prepared by published methods and trifluoroacetonylated by the usual procedure. The ^{19}F nmr spectrum of $(\alpha_2^{IIICN}\beta_2{}^{O_2})^{TFA}$ was identical to that of Hb^{TFA}-O_2. The oxygen affinity of the hybrid is very high, and complete deoxygenation could not be achieved by repeated application of the usual technique. The deoxygenated species which was produced had a chemical shift 30 cps upfield of the liganded peak,

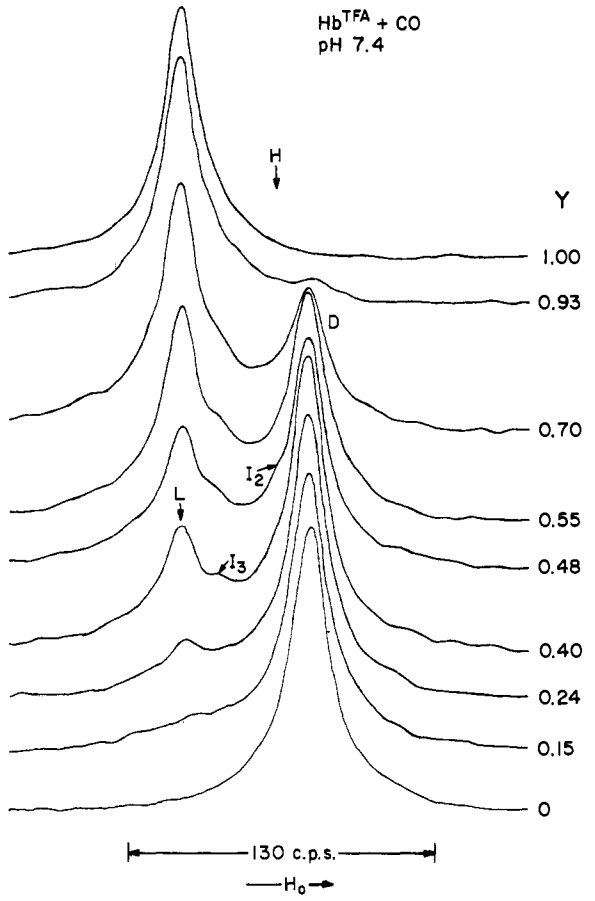

FIGURE 3: Nmr spectrum of Hb^{TFA} as a function of increasing fraction bound to carbon monoxide (Y). Peak D is the absorbance due to Hb^{TFA}-deO_2; L is due to Hb^{TFA}-$(CO)_4$; I_2 and I_3 are due to partially liganded intermediate species. The arrow "H" marks the chemical shift position of the high-affinity (β^*) unliganded form.

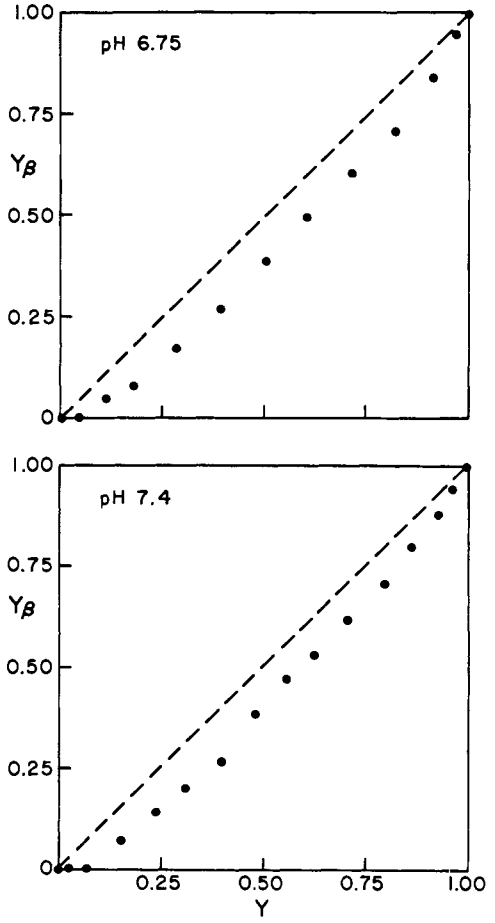

FIGURE 4: The increase in fractional ligation of β chains (Y_β) *vs.* the total ligation (Y). $Y_\beta = L + 1/2I_3$.

which was 20 cps downfield of the normal position of Hb^{TFA}-deO_2 at the pH used (6.75). Addition of a twofold excess of DPG to the solution caused the deoxygenated species peak to shift upfield by 20 cps. (Addition of DPG to Hb^{TFA}-deO_2 produces a similar upfield shift (Huestis and Raftery, 1972a) so that in the presence of DPG the two would still be ~15 cps apart.) The absence of any signal at the chemical shift position characteristic of Hb^{TFA}-IIICN indicated that no equilibration of heme ligands had occurred. Hence the species observed at +30 and +50 cps from the liganded peak position was $\alpha_2^{IIICN}\beta_2^{deO_2}$, showing that the chemical shift of the fluorine probe moiety reflects the ligand state and conformation of the β chains.

Comparison of Y with Y_β. The relative magnitudes of the nmr peaks reflect the fractional ligation of the β chains (Y_β). By measuring the visible absorbance change of the sample for each ligand pressure, the total fraction of liganded subunits (Y) corresponding to Y_β was obtained. After the visible absorbance had begun to indicate ligand binding, a lag ensued before liganded species appeared in the nmr spectrum. The fully liganded peak (L) and the intermediates reached detectable concentrations at about $Y = 0.15$. The combined magnitude of liganded nmr peaks lagged behind Y through most of the fractional ligation range. The ligand binding curves (Y and Y_β *vs.* log P_{CO}) obtained from visible and nmr spectra are compared in Figure 4. In Figure 4, the relative increase in Y_β is compared with Y, demonstrating again the lag in appearance of liganded β chains. (Calculation of Y_β from the magnitudes of the nmr peaks is discussed below.)

Discussion

The classical models for ligand binding to hemoglobin treat the α and β chains as equivalent binding sites. More recently, kinetic (Olson and Gibson, 1972) and structural (Perutz, 1970) evidence has been reported which indicates that the chains are nonequivalent and bind ligands in a preferential order. Previous work in this laboratory showed that in the presence of DPG, α chains preferentially bind the ligand *n*-butyl isocyanide (Huestis and Raftery, 1972c). In the nmr studies described in this communication, preferential binding of carbon monoxide to α chains has been observed in the presence of a 2:1 molar excess of DPG. Subsequent studies showed that oxygen also binds preferentially to α chains under these conditions, and that carbon monoxide exhibits preferential binding to α chains even in the absence of organic phosphates (W. H. Huestis and M. A. Raftery, manuscript in preparation). In a recent report, Lindstrøm and Ho (1972) described preferential binding of oxygen to α chains, but only in the presence of large excesses of DPG (15:1). They did not report differences in affinities of the chains with smaller amounts of DPG, nor was differential affinity for carbon monoxide observed under any conditions. Since the difference in binding to α and β chains is quite small (~10%) throughout the ligation range, it is possibly difficult to detect by nmr methods other than those described here.

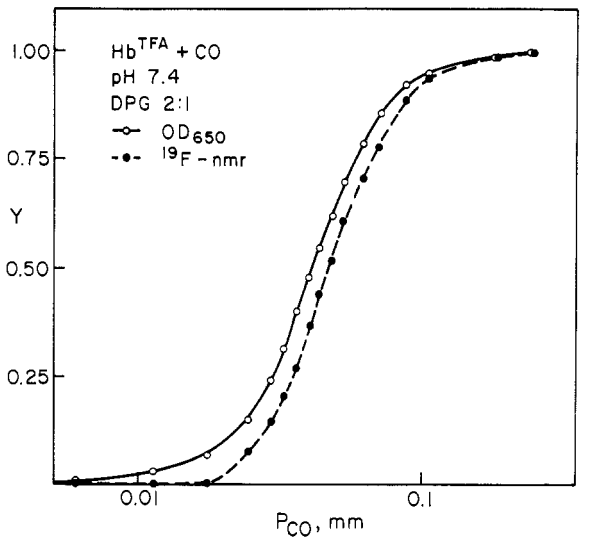

FIGURE 5: Carbon monoxide binding curves observed concurrently in visible and nmr spectra: (○) Y; (●) Y_β. $Y_\beta = L + 1/2I_3$.

First Stages of Ligand Binding. Partially liganded Hb^{TFA} solutions exhibited nmr absorptions in positions corresponding to Hb^{TFA}-deO_2 and Hb^{TFA}-$(CO)_4$, in addition to two intermediate signals (Figure 3). The liganded and intermediate signals appeared after the visible absorption change indicated that about 15% of the chains had bound a ligand (Figure 5). Experiments with the artificial intermediate $\alpha_2^{IIICN}\beta_2$, in which the α chains are locked in a liganded state but the β chains can be deoxygenated, showed that chemical shift changes in Hb^{TFA} reflect the ligation and conformation of the β chains. Thus the absence of changes in the nmr spectrum at low fractional ligation indicates that the first ligands must bind preferentially to α chains.

This result conflicts with the interpretation made by Gibson and coworkers (1972) of kinetic data from the binding of *n*-butyl isocyanide to hemoglobin. The results of nmr experiments on *n*-butyl isocyanide binding conducted in this laboratory (Huestis and Raftery, 1972c) were consistent with the conclusions drawn from the carbon monoxide binding experiments.

Identification of Intermediate Species. Analysis of the compositions of species producing distinct nmr absorptions yields information on the subsequent stages of ligand binding. The number of ligands bound to each species was determined from log–log plots of the carbon monoxide pressure *vs.* the ratio of magnitudes of each pair of peaks. The line obtained in each case has a slope equal to the average number of ligand molecules by which the species differ. Thus, for example, at pH 6.75 the L/D plot exhibited a slope of 4.08 by a least-squares fit (Figure 2), indicating that the majority of molecules in peak L contains four ligands more than those in peak D. Therefore the two large components arise from deoxy- and fully carboxygenated hemoglobin. The intermediate peak near L yielded an I_3/D slope of 3.22 and an L/I_3 slope of 0.9. Hence "I_3" arises from hemoglobin molecules which contain three ligands. (The intercept of the L/I_3 plot yields the equilibrium constant for the final ligand binding step, which is approximately 100 mm^{-1}. This number is approximately the equilibrium constant determined by Roughton (1953) from kinetic data.) The intermediate 15 cps downfield of D, I_2, yielded an I_2/D slope of 1.7 and an L/I_2 slope of 2.1. Thus I_2 represents molecules which have bound two ligands.

The chemical shift positions of the intermediates permit tentative characterization of the molecular species involved. For example, the chemical shift of I_2 is independent of ligand pressure; hence exchange between I_2 and species containing different numbers of ligands is slow on the nmr time scale. Since the chemical shift position of I_2 is different from that of L, and is pH dependent like that of D, it is reasonable to suppose that at least one unliganded β chain is present. Indeed, under the same conditions, the chemical shift of I_2 is identical with that of the artificial intermediate $\alpha_2^{IIICN}\beta_2^{deO_2}$ and responds to addition of a 2:1 excess of DPG in the same way. These factors suggest that I_2 is $\alpha_2^{CO}\beta_2^{deO_2}$. This would require that the second ligand, like the first, binds preferentially to an α chain.

Similarily, the chemical shift of I_3 indicates that its unliganded subunit is a β chain. The position of I_3 is independent of ligand pressure and of pH, and it shifts *down*field on addition of DPG. These factors indicate that exchange with species of different ligand content is slow, that DPG does not bind in the same way or to the same extent as to I_2 or D, and that the unliganded β chain resembles liganded hemoglobin more than deoxyhemoglobin in certain conformational features near Cys-β93 (Huestis and Raftery, 1972b). Thus, I_3 arises from a species $\alpha_2^{CO}\beta^{CO}\beta^*$, where β^* is an unliganded form having a conformation different from that of either liganded or deoxyhemoglobin. Having thus identified I_2 and I_3, the fraction of ligated β chains (Y_β) can be calculated as the sum of L (containing two liganded β chains) and one half of I_3 (containing one liganded β chain).

Some order may be introduced into this assortment of molecular species if we point out certain relationships between their chemical shifts. Carboxy- and deoxyhemoglobin absorb 60 cps apart at pH 6.75. As the pH is increased, the resonance position of deoxyhemoglobin moves downfield until, at pH 8.5, it appears only 30 cps upfield of carboxyhemoglobin (Huestis and Raftery, 1972a). This chemical shift position is almost exactly that of $\alpha_2^{IIICN}\beta_2^{deO_2}$ and I_2 when DPG is absent. Both high pH and partial ligation confer extremely high ligand affinities on deoxy subunits; hence the chemical shift of these three hemoglobin species is characteristic of β chains of high ligand affinity. The chemical shift of I_3, 15 cps downfield of this "high affinity" position and independent of pH, indicates that the unliganded β chain is in yet another conformation distinct from other unliganded β chains.

Conclusion

^{19}F nmr studies of the species present in partially liganded hemoglobin solutions have permitted comparison of overall fractional saturation to the fractional saturation of β chains. The results indicated that α chains preferentially bound carbon monoxide in the presence of a 2:1 molar excess of DPG, at pH 6.75 and 7.40. Further examination of the intermediate species present in partially carboxygenated solutions indicated that unliganded β chains in tetramers containing two or three ligands exhibit chemical shifts characteristic of "high-affinity" deoxy forms, intermediate between the chemical shift positions of deoxy- and fully liganded hemoglobin.

Acknowledgment

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A Comparison of the Functional Properties of Human Hemoglobin A and Its (β-93)-Trifluoroacetonylated Derivative†

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ABSTRACT: pH and salt effects on the oxygenation equilibrium of human hemoglobin A and its trifluoroacetonylated derivative Hb^{TFA} have been studied. The binding of 2,3-diphosphoglycerate to both hemoglobins has also been studied as a function of ligand saturation. The striking resemblance of these properties of Hb-A and Hb^{TFA} allows meaningful ^{19}F nmr experiments using TFA as a probe; in both instances, the Hill coefficient was found to be invariant with both pH and ionic strength. The implications of this result are discussed in relation to the cooperative mechanism.

Trifluoroacetonylated hemoglobin (Hb^{TFA}) has been used in this laboratory for studies of conformational processes accompanying the binding of ligands and allosteric effectors (Huestis and Raftery, 1972a–c, 1973). The pertinence of these findings to the mechanism of native hemoglobin depends on the degree to which introduction of the trifluoroacetonyl group at cysteine-β93 perturbs native functions. This communication reports a systematic comparison of the functional properties of Hb^{TFA} and native hemoglobin. The effects of pH and ionic strength on the oxygenation equilibria were examined, and the release of DPG on ligand binding was compared using ^{31}P nuclear magnetic resonance (nmr).

The studies of oxygenation equilibria also yielded reasonable explanations for some conflicting reports on effects of pH and ionic strength on the Hill coefficient. Antonini and coworkers (Antonini *et al.*, 1962) have reported that cooperativity decreases at low salt concentrations, but is invariant with pH in the range 6–9 in 0.1 M salt. In contrast, Kilmartin and Hewitt (1971) report a decline in cooperativity with increasing pH, and Benesch *et al.* (1969) have suggested that the decrease of cooperativity observed at low ionic strength is an artifact resulting from partial removal of DPG. The results reported here provide consistent explanations of these observations.

Experimental Section

Materials. 3-Bromo-1,1,1-trifluoropropanone was obtained from Peninsular Chemresearch Inc. 2,3-Diphosphoglyceric acid was obtained as the pentacyclohexylammonium salt from Calbiochem and converted to the free acid by shaking with Dowex 50-X8 (H^+ form). Bis-tris was a product of Aldrich Chemical Co.

Human hemoglobin was isolated, purified, and trifluoroacetonylated as previously described (Huestis and Raftery, 1972a).

Methods. Oxyhemoglobin concentrations were determined from their absorbance at 540 nm ($E_{1\%}^{1\,cm}$ 8.5) using a Gilford Model 240 spectrometer with Gilford Model 410 digital absorbance meter. pH measurements were made using Radiometer Copenhagen Model 26 pH meter. ^{31}P nmr spectra were recorded on a Varian XL-100 spectrometer modified to operate at 40 MHz, and with phosphorus Fourier transform capability. Spectra were obtained in 1000 pulses using an acquisition time of 0.7 sec.

For oxygenation studies, hemoglobin solutions of different pH's were prepared as follows: a suitable amount of the stock hemoglobin solution was put into an Amicon diaflow apparatus with a PM-10 membrane, diluted with the buffer of the desired pH, and concentrated; this process was repeated several times.

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§ Postdoctoral Fellow of the Sloan Foundation.
¶ National Institutes of Health Career Development awardee, 1972.

[1] Abbreviations used are: Hb-A, native human hemoglobin; Hb^{TFA}, trifluoroacetonylated human hemoglobin; DPG, 2,3-diphosphoglycerate; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol.