References

Adams, M. J., Ford, G. C., Kockoek, R., Lentz, P. J., McPherson, A., Rossman, M. G., Smiley, I. E., Schevitz, R. W., and Wonacott, A. J. (1970a), *Nature (London) 227*, 1098.

Adams, M. J., McPherson, A., Rossmann, M. G., Schevitz, R. W., and Wonacott, A. J. (1970b), *J. Mol. Biol. 51*, 31.

Catterall, W. A., Hollis, D. P., and Walter, C. F. (1969), *Biochemistry 8*, 4032.

Cohn, M., and Hughes, T. R. (1960), *J. Biol. Chem. 235*, 3250.

DiSabato, G. (1968), *Biochim. Biophys. Acta 167*, 646.

Griffith, P. R., Rye, M. J., and Alexander, J. G. (1970), *Can. J. Biochem. 48*, 947.

Ho, C., Magnuson, J. A., Wilson, J. B., Magnuson, N. S., and Kurland, R. J. (1969), *Biochemistry 8*, 2074.

Hollis, D. P. (1967), *Biochemistry 6*, 2080.

Hollis, D. P. (1969), *Org. Magn. Res. 1*, 305.

Huestis, W. H., and Raftery, M. A. (1971), *Biochemistry 10*, 1181.

Huestis, W. H., and Raftery, M. A. (1972), *Biochemistry 11*, 1648.

Jacobus, J. (1971), *Biochemistry 10*, 161.

Jardetzky, O., Wade, N. G., and Fischer, J. J. (1963), *Nature (London) 197*, 183.

Jardetzky, O., and Wade-Jardetzky, N. G. (1966), *J. Biol. Chem. 241*, 85.

Laforge, F. B. (1928), *J. Amer. Chem. Soc. 50*, 2477.

Moore, C. E., and Underwood, A. L. (1969), *Anal. Biochem. 29*, 149.

Murray, A., Foreman, W. W., and Langham, W. (1948), *J. Amer. Chem. Soc. 70*, 1037.

Sarma, R. H., Dannies, P., and Kaplan, N. O. (1968a), *Biochemistry 7*, 4359.

Sarma, R. H., and Kaplan, N. O. (1969a), *Biochem. Biophys. Res. Commun. 36*, 780.

Sarma, R. H., and Kaplan, N. O. (1969b), *J. Biol. Chem. 244*, 771.

Sarma, R. H., and Kaplan, N. O. (1970a), *Biochemistry 9*, 539.

Sarma, R. H., and Kaplan, N. O. (1970b), *Biochemistry 9*, 557.

Sarma, R. H., and Kaplan, N. O. (1970c), *Proc. Nat. Acad. Sci. U. S. 67*, 1375.

Sarma, R. H., Moore, M., and Kaplan, N. O. (1970), *Biochemistry 9*, 549.

Sarma, R. H., Ross, V., and Kaplan, N. O. (1968b), *Biochemistry 7*, 3052.

Sarma, R. H., and Woronick, C. L. (1971), *Res. Commun. Chem. Pathol. Pharmacol. 2*, 177.

Zatman, L. J., Kaplan, N. O., and Colowick, S. P. (1953), *J. Biol. Chem. 200*, 197.

Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1954), *J. Biol. Chem. 209*, 467.

A Study of Cooperative Interactions in Hemoglobin Using Fluorine Nuclear Magnetic Resonance†

Wray H. Huestis‡ and M. A. Raftery*

ABSTRACT: The interaction of hemoglobin with heme ligands and allosteric effectors has been studied by fluorine nuclear magnetic resonance spectroscopy. For this purpose hemoglobin has been labeled covalently with 3,3,3-trifluoroacetonyl groups on the β-chain cysteine residues at position 93. Oxidation of the heme iron, as well as binding heme ligands, protons, and diphosphoglycerate to the labeled hemoglobin, produces significant changes in the ^{19}F nuclear magnetic resonance spectrum of the label. Interpreted in light of crystal structure data and the chemical properties of hemoglobin, these changes yield information on tertiary structural changes accompanying allosteric interactions.

The allosteric properties of hemoglobin have long been assumed to result from conformation changes in the protein which occur on oxygenation. Structural differences between liganded and unliganded hemoglobin are suggested by an extensive array of evidence from crystal forms (Haurowitz, 1938), optical rotation (Briehl, 1962), carboxypepitdase digestion rates (Zito *et al.*, 1964), stability and solubility changes (Cohn and Edsall, 1943), dye binding (Antonini *et al.*, 1963), and rates of reaction with thiol-specific reagents (Riggs, 1961). The work of Perutz and coworkers on the crystal structures of oxy- (Perutz *et al.*, 1968) and deoxyhemoglobin (Muirhead and Greer, 1970) has elucidated differences between the two forms and suggested mechanisms by which the conversion from one to the other could occur in a cooperative manner. From these crystal structures, Perutz (1970b) has further proposed mechanisms by which allosteric effectors (protons and orgainc phosphates) could influence the properties of hemoglobin.

The objective of this investigation was to examine the allosteric interactions of hemoglobin on a molecular level by studying the ^{19}F nuclear magnetic resonance (nmr) spectrum of a small fluorinated probe molecule strategically attached to the protein. The probe used was a 3,3,3-trifluoroacetonyl group placed on the side chain of cysteine-β93, at which position

† Contribution No. 4289 from the Church Laboratory of Chemical Biology, California Institute of Technology, Pasadena, California 91109. *Received August 9, 1971.* Supported by U. S. Public Health Service Grant GM-16424. A preliminary report of this work was presented at the Cold Spring Harbor Symposium of Quantitative Biology, June 3–10, 1971.

‡ National Science Foundation Predoctoral Fellow, 1970–1971.

other workers have introduced electron spin resonance probes (McConnell *et al.*, 1969). Binding of heme ligands, protons, and diphosphoglyceric acid to labeled hemoglobin produced changes in the ^{19}F nmr spectrum of the trifluoroacetonyl group which, interpreted in light of crystallographic results, provide evidence on the mechanisms of these interactions.

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. 5,5′-Dithiobis(2-nitrobenzoic acid) was the product of Aldrich Chemical Co. *S*-Trifluoroacetonylmercaptoethanol ($HOCH_2CH_2SCH_2COCF_3$, I) was synthesized by direct combination of β-mercaptoethanol with 3-bromo-1,1,1-trifluoropropanone at room temperature. The white crystalline product was recrystallized fron benzene–ligroin and characterized by its infrared and ^1H nmr spectra.

Methods. Hemoglobin concentrations were determined from their absorbance at 540 nm ($E^{1\,cm}_{1\%}$ = 8.5 at 540 nm) using a Gilford Model 240 spectrophotometer. pH measurements were made using a Radiometer Copenhagen Model 26 pH meter. ^{19}F nmr spectra were recorded using Varian Models HA-100 and XL-100-15 spectrometers modified to operate at 94.1 MHz. Both were supplemented by a Fabritek Model 1061 computer of average transients. Spectrum accumulation times were reduced by use of 12-mm outer diameter sample tubes. Resonance positions were measured routinely from trifluoroacetic acid in a capillary tube, or from the H_2O lock signal. Additional controls for bulk diamagnetic susceptibility effects were made by measuring shifts from *S*-trifluoroacetonylmercaptoethanol as an internal standard.

Isolation of Human Hemoglobin. Erythrocytes from freshly drawn citrated blood were washed three times with 0.9% NaCl solution. The packed cells were lysed by addition of 1 volume of distilled water and 0.4 volume of toluene. After being shaken for 4 min, the mixture was centrifuged. The clear hemolysate was removed and dialyzed against distilled water. All steps were carried out at 4°. Hemoglobin stock solutions were stored at 4° under argon and were used within 10 days.

Preparation of ^{19}F-Labeled Hemoglobin. A solution of 600 mg of hemoglobin (1.86×10^{-5} mole of Cys-β93) in 6 ml of H_2O was added to 6 ml of sodium phosphate buffer (0.4 M, pH 7.15). 3-Bromo-1-trifluoropropanone (50 μl, 28 mg, 4.6×10^{-5} mole) was added and the solution was stirred at room temperature for 30 min, during which time the pH was maintained at 7.15 by addition of 0.5 M sodium hydroxide. A precipitate was removed by centrifugation, and the reaction mixture was subjected to gel filtration on a Bio-Gel P-2 column (2.5×25 cm, eluting solvent 0.1 M NaCl) to remove excess reagent, organic phosphates, and phosphate. For nmr studies the hemoglobin solution was concentrated to 10% by ultrafiltration employing an Amicon apparatus.

Sulfhydryl group determinations were carried out by the method of Ellman (1959). Oxygen equilibria and Hill coefficients were determined for Hb and Hb^{TFA}[1] by a modification of the procedure of Riggs and Wohlbach (1956). Equilibria were determined at 25° in 0.1 M sodium phosphate (pH 7.0).

Deoxyhemoglobin (Hb^{TFA}) was prepared from oxyhemoglobin (Hb^{TFA}-O_2) by repeated washing with nitrogen in a tonometer. Methemoglobin (Hb^{TFA}-III) was prepared by addition of $K_3Fe(CN)_6$ (600 μl of 0.1 M solution, 6×10^{-5} mole) to oxyhemoglobin (400 mg, 2.4×10^{-5} mole of Fe^{II}) in 10 ml of sodium phosphate buffer (0.01 M, pH 7.0). The solution was allowed to stand at room temperature for 1 hr, then the Hb^{TFA}-III was purified by passage through a Bio-Gel P-2 column (2.5×25 cm) with 0.1 M NaCl as eluting solvent. Cyanmethemoglobin (Hb^{TFA}-III CN) was prepared by the same procedure, with a 100-fold excess of KCN (in 1 ml of H_2O, pH 7) being added at the same time as the $K_3Fe(CN)_6$. Determinations of Hb^{TFA}-III and Hb^{TFA}-III CN were carried out by the method of Tomita *et al.* (1968).

Carboxyhemoglobin (Hb^{TFA}-CO) was prepared by washing Hb^{TFA}-O_2 with carbon monoxide in a tonometer.

Nmr Spectral Measurements. Solutions used for nmr studies contained 300 mg of hemoglobin in 3 ml of 0.1 M NaCl solution. pH adjustments were made by slow addition of 0.1 M HCl or NaOH, with stirring. The pH of each solution was measured before and after the spectrum was recorded. Deoxyhemoglobin solutions were transferred from tonometer to argon-filled nmr tubes with a syringe. DPG, 0.16 M in H_2O (pH 7.0), was added to hemoglobin solutions in aliquots adequate to ensure 90% saturation at each pH, while avoiding oversaturation which might have resulted in nonspecific binding (Garby *et al.*, 1969). Molar ratios of DPG:hemoglobin required to meet these criteria were calculated from the binding data of Benesch and Bensch (1970) and Garby *et al.* (1969).

Results

Characterization of Trifluoroacetonylated Hemoglobin. The extent of modification of the exposed SH groups of hemoglobin was determined spectrophotometrically, using the 5,5′-dithiobis(2-nitrobenzoic acid) method of Ellman (1959). Hb^{TFA}-O_2 was found to contain no exposed SH groups, as compared to 1.9 moles/mole of tetramer for native Hb-A.

The oxygen equilibrium curves of Hb and Hb^{TFA} are shown in Figure 1. The functional effect of introduction of the trifluoroacetonyl groups is not great; oxygen affinity was decreased only slightly, and the Hill coefficient was reduced from 2.7 to 2.5.

Nmr Spectra of Trifluoroacetonylated Hemoglobin. The spectrum of Hb^{TFA}-O_2 consisted of a singlet which appeared 483 cps upfield of trifluoroacetic acid.[2] Deoxygenation to Hb^{TFA} produced a shift to higher field of 52 cps in the ^{19}F resonance, which appeared at +535 cps. For Hb^{TFA}-III(methemoglobin) the resonance was found at +525 cps; for Hb^{TFA}-CO and Hb^{TFA}-III CN resonances appeared at +470 and +460 cps, respectively (Figure 2).

Effect of Phosphate and Diphosphoglyceric Acid on Spectra of Hb^{TFA}. Addition of diphosphoglyceric acid (DPG) to Hb^{TFA} shifted the ^{19}F resonance to higher field (Figure 3). Below pH 7, the presence of 0.5 mole equiv of DPG shifted the resonance to +540 cps, and 1.0 mole equiv of DPG shifted it to +547 cps—a total of +12 cps. Further, in 0.1 M phosphate buffer the Hb^{TFA} signal appeared at +540 cps in the absence of DPG. The presence of DPG or phosphate did not

[1] Abbreviations used are: DPG, diphosphoglycerate; PMB, *p*-mercuribenzoate; TFA, trifluoroacetonyl; Hb, deoxyhemoglobin A, Hb-O_2, oxyhemoglobin; Hb-CO, Hb-III, Hb-III CN, carboxy-, met-, and cyanmethemoglobin, respectively; and Hb^{TFA}, trifluoroacetonylated deoxyhemoglobin.

[2] Shifts are given relative to trifluoroacetic acid, for Hb^{TFA} 1.5×10^{-3} M in 0.1 M NaCl (pH 6.75).

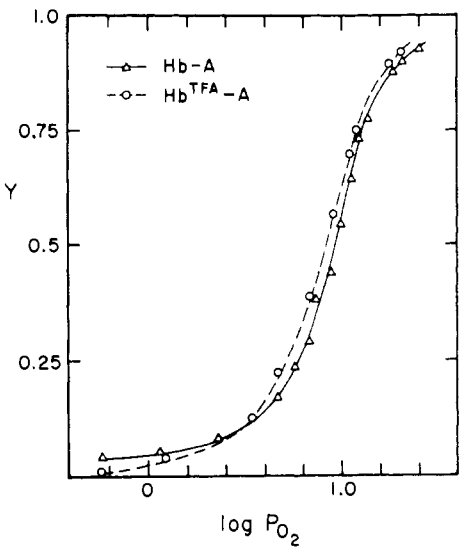

FIGURE 1: Oxygen equilibrium curves for Hb and Hb^{TFA} at 25° in 0.1 M sodium phosphate (pH 7.0).

change the position of the Hb^{TFA}-O_2 resonance when other salts (*e.g.*, 0.1 M NaCl) were present.

pH Dependence of the ^{19}F Nmr Spectrum. The effect of pH on the chemical shift of labeled hemoglobin is shown for various ligands in Figure 4. The position of the ^{19}F resonance of Hb^{TFA}-O_2 was constant in the pH range 6.0–8.5. The resonance positions for Hb^{TFA}-CO and Hb^{TFA}-III CN also were essentially constant for the pH range 7.0–8.0. The chemical shift of Hb^{TFA} underwent a signficant change (~40 cps) in this pH range, and a plot of chemical shift change *vs.* pH suggests the influence of an ionizable group of pK_a = 7.4.

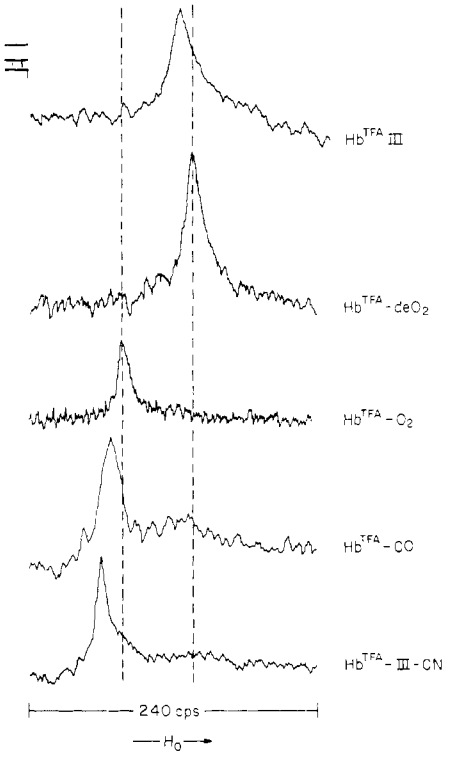

FIGURE 2: ^{19}F nmr spectra of trifluoroacetonylated hemoglobin for various ligand states.

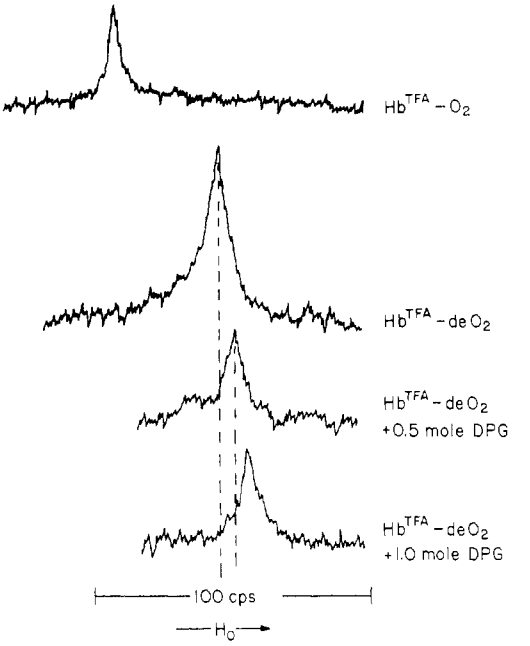

FIGURE 3: ^{19}F nmr spectra of trifluoroacetonylated deoxyhemoglobin in the presence of DPG, in 0.1 M NaCl at pH 6.75.

This pH effect was again evident in the presence of DPG (Figure 5); the titration curve showed an apparent pK_a of ~7.4, but was somewhat steeper. The resonance position of Hb^{TFA}-III also changed by ~30 cps with pH, apparently reflecting the titration of a group of pK_a = 7.8 (Figure 6).

To examine possible direct effects of pH on the ^{19}F probe moiety, the ^{19}F chemical shift of a model compound, I was studied as a function of pH. As is shown in Figure 4, the ^{19}F resonance position was invariant from pH 6.0 to 8.0, above which a small downfield shift was apparent which was not observed in spectra of protein-bound probes.

Discussion

In an effort to study mechanisms of transitions in hemoglobin on a molecular level, we have covalently attached a small fluorinated probe group to the protein and studied its ^{19}F nmr spectrum as a function of binding of ligands and allosteric effectors. For such an approach to be useful, the probe moiety must be positioned strategically so that its magnetic environment will be affected by the processes of interest, but so that it perturbs the native properties of the protein as little as possible.

The probe used in these studies was a trifluoroacetonyl group attached to the protein by reaction with the thiol at cysteine-β93. Several types of modifications at this position have been studied extensively, and it has been found that some modifying groups, particularly bifunctional (Benesch and Benesch, 1961; Moffat *et al.*, 1971) or rather large (McConnell *et al.*, 1969; Moffat, 1971) ones, profoundly alter the functioning of the protein. However, Riggs (1961) showed that carboxymethylation of this cysteine produced minimal changes in the protein's allosteric properties—oxygen affinity was increased, particularly at low degrees of oxygenation, but the Hill coefficient and the Bohr effect were not greatly altered. Trifluoroacetonylation of Cys-β93 produced even smaller perturbations of these properties; Hb^{TFA} exhibited an oxygen affinity almost identical with that of native Hb A and a Hill coefficient of 2.5 (Figure 1).

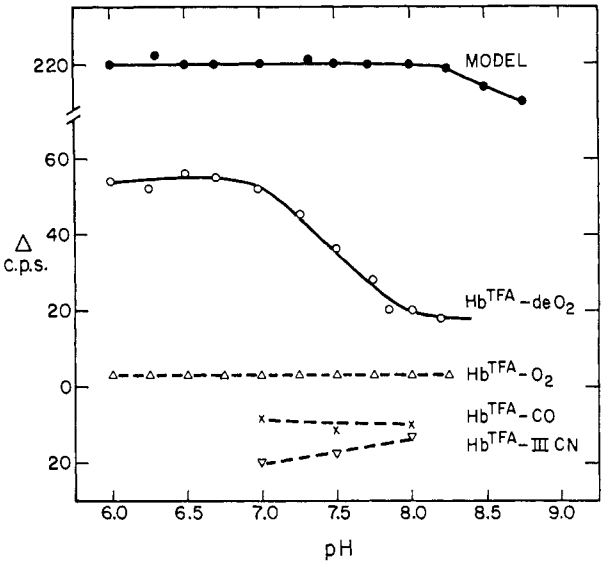

FIGURE 4: Chemical shift of trifluoroacetonylated hemoglobin as a function of pH, for various ligand states (○,△,▽,×) and for *S*-trifluoroacetonyl-β-mercaptoethanol (●).

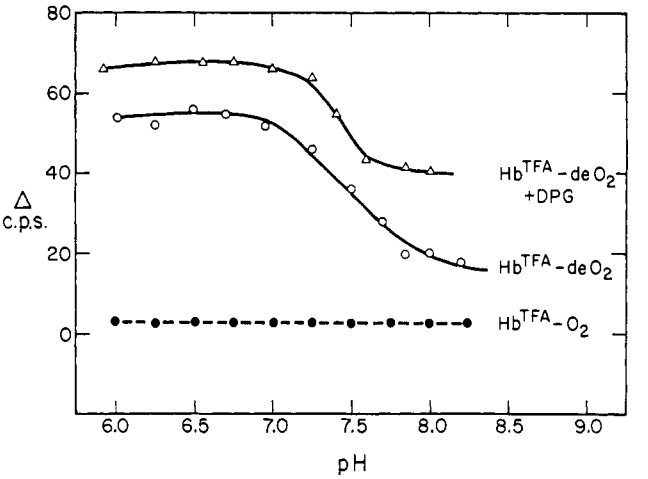

FIGURE 5: Chemical shift of trifluoroacetonylated hemoglobin as a function of pH: (●) Hb^{TFA}-O$_2$ in presence or absence of DPG; (○) Hb^{TFA}, (△) Hb^{TFA} and DPG.

The sensitivity of hemoglobin functions to alterations in this region suggests that Cys-β93 is a useful position for a conformation sensitive probe. It lies in the $\alpha_1\beta_2$ interface, next to His-β92 (the proximal heme ligand) and Asp-β94, which forms a salt bridge to the imidazole of His-β146 in deoxyhemoglobin (Perutz, 1970b). Thus, depending on its length, an nmr probe attached to Cys-β93 could yield information on tertiary and/or quaternary structural changes involved with the His-β146 salt bridges, or could sense other quaternary structural changes which altered orientations or distances in the $\alpha_1\beta_2$ contact region. The α chain is about 10 Å away from Cys-β93 and the trifluoroacetonyl group is only about 4 Å long, so direct influences on its environment should result primarily from its own β chain.

Oxy- and Deoxyhemoglobin^{TFA}. The nmr spectra of liganded trifluoroacetonylated hemoglobin showed that the environment of the probe group was similar for several ligands (Hb^{TFA}-O$_2$, Hb^{TFA}-CO, and Hb^{TFA}-III CN). The chemical shifts of these derivatives differed only slightly, and did not show a large pH dependence. However, small differences in chemical shifts were observed for all liganded species indicating that although their structures are similar in the region of the probe they are not identical. Deoxyhemoglobin exhibited quite different behavior; its probe resonance appeared 52 cps to higher field of Hb^{TFA}-O$_2$ and the resonance position was pH dependent. A plot of chemical shift *vs.* pH for Hb^{TFA} (Figure 4) had the appearance of a titration curve of an ionizable group of pK = 7.4. The observed change in chemical shift could be due to either a direct charge effect from the ionizable group, or an ionization-induced conformation change in the protein which produces a change in the magnetic environment of the ^{19}F probe.

A useful consideration in the analysis of this result is the absence of any pH dependence for the chemical shift of Hb^{TFA}-O$_2$. This could be interpreted to mean that an ionization which occurs at pH 7.4 in Hb occurs at very much higher or lower pH in Hb-O$_2$, or that a pH-dependent conformational process occurs in the pH range 6–8.5 in Hb but not in Hb-O$_2$, or that an ionizable group or ionization-induced conformational process occurs in the immediate vicinity of the probe in Hb, but that a conformational change occurring on oxygenation removes the groups involved too far away from the probe for it to experience the change.

Distinguishing among these alternatives would be difficult without knowledge of the ligand-dependent conformational processes which occur near Cys-β93. According to Perutz's analyses of the crystal structures and studies on spin-labeled hemoglobins (Deal *et al.*, 1971), the principle structural changes occurring near Cys-β93 involve the position of the carboxy-terminal residues of the β chains (Figure 7). In deoxyhemoglobin, the terminal histidine-β146 NC is doubly bound to the protein by salt bridges, an interchain link from its α-carboxyl group to lysine-α40 and an intrachain link from its imidazole to aspartate-β94. The formation of these bonds requires that tyrosine-β145 occupy a space between the F and G helices of the β chain. According to Perutz (1970b), ligand binding produces changes in the conformation of the β chain which include closing of the F–G pocket, forcing the tyrosine out and destabilizing the two salt bridges of His-β146. In addition, the quaternary structural change on oxygenation leads to breaking of the histidine carboxyl salt link to the α chain. In liganded crystalline hemoglobin,

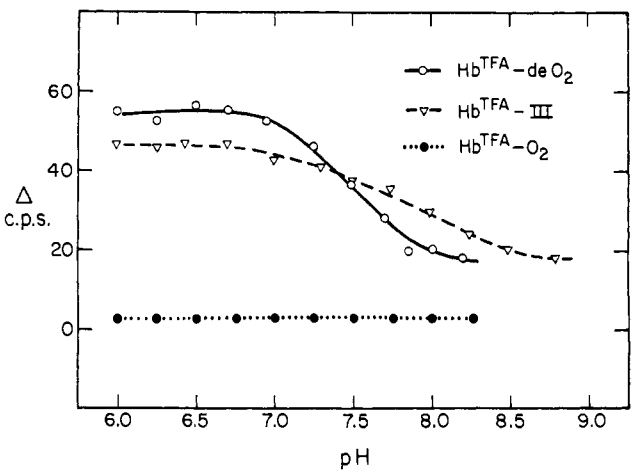

FIGURE 6: Chemical shift of trifluoroacetonylated hemoglobin as a function of pH.

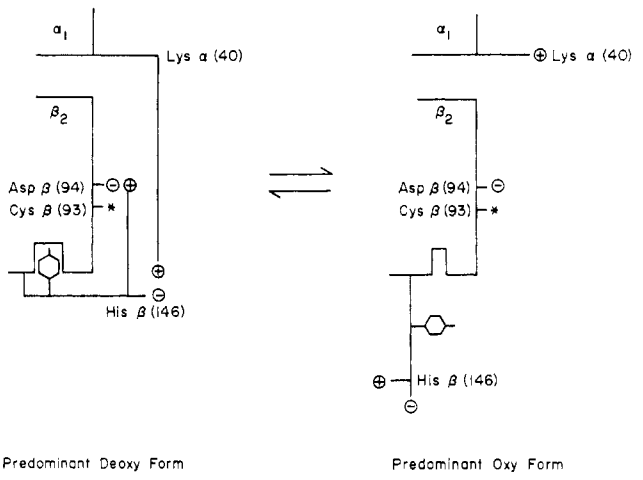

FIGURE 7: Schematic representation (after Perutz, 1970b) of the conformational equilibrium of the C terminus of the β chain, showing effects of pH and ligand binding in the $\alpha_1\beta_2$ contact region. The position of the ^{19}F probe is indicated by *.

these bonds are broken so that the carboxy terminal moves freely on the outside of the β subunit. Perutz points out, however, that this process should be regarded not as a transition between two fixed states, but as the shifting of the position of a dynamic equilibrium. Indeed, evidence from spin-label studies (Deal *et al.*, 1971) indicates that both "bound" and "free" conformations can occur in both oxy- and deoxyhemoglobin, depending on conditions of pH and ionic strength.

It seems likely that the chemical shift change of the ^{19}F probe (oxy → deoxy) reflects the shifting of this conformational equilibrium. In deoxyhemoglobin, the imidazole of His-β146 is predominantly bound to Asp-β94 (~4 Å away from Cys-β93), where its charge and/or its ring current could influence the magnetic environment of the ^{19}F probe. As ligand binding shifts the equilibrium in favor of the "free" state, the imidazole no longer lies near the ^{19}F probe. The pH dependence of the chemical shift in Hb^{TFA} implicates an ionization process of $pK_a = 7.4$ in this magnetic environment change, which is likely to be the ionization of the salt-bridged imidazolium side chain of His-β146. Some caution should be exercised in interpreting the chemical shift on Hb^{TFA} above pH 7.4 as a return to the "free" conformation by His-β146, since the salt bridge between the His-β146 carboxyl and Lys-α40 is still possible. It is likely that the breaking of the imidazole salt bridge in Hb^{TFA} shifts the equilibrium in favor of the "free" terminus position (as is reflected in higher ligand affinity at higher pH's), but no unequivocal statement can be made about the average conformation since the chemical shift change could be due merely to the removal of the influence of the imidazole's positive charge.

A search for other groups in the protein which might account for the observed environmental changes does not yield any likely candidates. The spin state of the iron atom is changed from high spin (paramagnetic) to low spin (diamagnetic) on ligand binding, but the spin state is not pH dependent in this case, and in deoxy β chains there is no room (Perutz, 1970b) near the heme for a ligand (such as H_2O in deoxy α chains) which might change with pH. Above pH 8, the chemical shift difference between oxy- and deoxyhemoglobin is only 12 cps, so any contribution from the paramagnetic iron, if at all present, is small.

Several other groups in the vicinity of Cys-β93 ionize in the physiological pH range, but for various reasons none is likely to produce the observed pH dependence. His-β92, the proximal heme-linked histidine, is the neighbor of Cys-β93 in the F helix. Its pK would be expected to be lowered substantially due to its bond to the iron (a pK_a of 5.1–5.3 has been assigned to this residue in methemoglobin (George and Hanania, 1952)), so conformation or magnetic field changes due to this residue would not occur around pH 7.4. His-β143 undergoes a substantial environment change on ligand dissociation which brings it near to Cys-β93, and which might cause its pK_a to change. However, His-β143 is unlikely to be the group which affects Cys-β93's environment, since the binding of DPG, which raises the pK_a of His-β143 (Perutz, 1970a), does not change the pH dependence of the fluorine probe's chemical shift (Figure 5). His-β146 is the most likely source of the environment change, implying that its pK_a in deoxyhemoglobin is 7.4. The pK_a of His-β146 has not been determined for oxyhemoglobin, but since the C terminus of the oxy β chain is predominantly in the free conformation, the imidazole pK would be expected to be similar to that observed in small peptides terminating in histidine (6.7–6.8) (Perrin, 1965). If so, deoxygenation is accompanied by a pK_a rise of 0.6–0.7 pH unit for this residue. Such a change has been suggested as the source of half of the alkaline Bohr effect (Wyman and Allen, 1951; Kilmartin and Wootton, 1970).

Diphosphoglyceric Acid Binding to Hb^{TFA}. DPG binds to unliganded but not liganded hemoglobin, decreasing the oxygen affinity and increasing cooperativity without altering the Bohr effect (Benesch *et al.*, 1969; Tyuma and Shimizu, 1970; Tyuma *et al.*, 1971). Extensive studies on this interaction have been conducted by several investigators, and there is general agreement that the "specific" DPG binding site is between the β chains in the cluster of positive charges formed by lysines-β82, histidines-β143, and the amino groups of valine-β1. The increased cooperativity and decreased oxygen affinity produced by this binding have been attributed (Perutz, 1970b) to stabilization of the deoxy structure by the salt bridges which DPG forms between the β chains.

In the presence of a 1:1 molar ratio of DPG, the ^{19}F nmr signal of Hb^{TFA} shifted to higher field by ~12 cps in the pH range 6–8, while exhibiting the same sort of pH dependence observed in phosphate-free Hb^{TFA} (Figures 3 and 5). An exact interpretation of this shift is difficult. A conformation change probably has occurred, as evidenced by the change in chemical shift at all pH values, but since no crystallographic evidence is yet available on the Hb·DPG complex, the exact nature of the change is not clear. An interesting aspect of the pH dependence of the Hb^{TFA}·DPG spectrum is the reproducible steepness of the titration curve. This behavior suggests that in the presence of DPG some sort of cooperative interaction is influencing the ionization of His-β146. Since the state of this ionization is important in the maintenance of the tertiary deoxy structure (Perutz, 1970b), the observed shortening of the deprotonation range could be a reflection of the cooperativity enhancement produced by DPG.

TFA-Methemoglobin. The pH-dependent phenomena discussed above do not appear in the spectrum of oxyhemoglobin, as is shown in Figure 4. This would be expected if the His-β146 ionization is causing the pH dependence, since the equilibrium position of the C terminus is removed from the vicinity of Cys-β93 on oxygenation. It is somewhat surprising to find that the chemical shift of methemoglobin (Hb^{TFA}-III) is pH dependent, for most previous evidence (dye binding, crystal form) indicates that Fe^{III} hemoglobin is similar to liganded Fe^{II} hemoglobin at least in quaternary structure. But the ^{19}F nmr signal of Hb^{TFA}-III lies 42 cps upfield of the

signal of Hb^{TFA}-O_2 (only 10 cps away from the Hb^{TFA} signal), and its chemical shift exhibits a substantial pH dependence (Figure 6). Since evidence already cited suggests that the nmr probe is influenced primarily by conformation changes within the β chain, its behavior in Hb^{TFA}-III suggests that at least in this region of the protein Hb-III differs from Hb-O_2 in tertiary structure. The X-ray structure work does not yield precise information on this point, since the oxyhemoglobin crystals used contained some undetermined quantity of methemoglobin throughout collection of the X-ray data (Perutz, 1970b). The structure of oxyhemoglobin presently available may have a tertiary structure intermediate between the native structures of oxy- and methemoglobin, if indeed they are different. Uncertainty as to the native conformation of the Cys-β93–His-β146 region may have been introduced by the use of PMB on Cys-β93 in two of the three heavy-metal derivatives of oxyhemoglobin. Cys-β93–PMB hemoglobin exhibits decreased cooperativity and Bohr effect. Hence it is difficult at present to assign specific causes to the nmr behavior of Hb^{TFA}-III on the basis of the crystal structure.

Several possible explanations are suggested by the apparent pK of 7.8 exhibited by the chemical shift change. Such a pK could be due to a histidine, such as residues 97, 143, 146, 92 (the proximal histidine), or 63 (the distal histidine) or an α-amino group, or it could be related to the ionization of the water molecule which is the distal heme ligand of methemoglobin. We can eliminate some, but not all, of these possibilities. The water ionization is accompanied by a spin-state change in the hemes, from high-spin paramagnetic (as in deoxyhemoglobin) to low-spin diamagnetic (as in oxyhemoglobin). However, the pK of the high- to low-spin transition is 8.15 (from magnetometric titration, Coryell *et al.*, 1937) to 8.3 (from optical difference titrations, Fabry *et al.*, 1969), substantially higher than the nmr spectrum change. Further, Hb^{TFA}, which is high spin paramagnetic over the entire pH range, shows the same small (~12 cps) shift from Hb^{TFA}-O_2 as does Hb^{TFA}-III above pH 8. Unless large compensatory changes occur, the paramagnetic heme field of Hb^{TFA}-III probably is not the origin of its nmr characteristics. Other ionizations directly associated with the methemoglobin heme have been observed by various techniques at pH 5.1–5.3 and 6.7–7.1, and attributed, respectively, to the proximal and distal histidines (Fabry *et al.*, 1969; George and Hanania, 1953). Neither of these ionizations can be solely responsible for the chemical shift change in Hb^{TFA}-III.

The general similarity of Hb^{TFA}-III's nmr characteristics to those of Hb^{TFA} suggests that it might be worthwhile to consider that the same group might be influencing the environment of the probe in both cases. This would require that the imidazole pK of His-β146 be raised from 7.4 to 7.8. In methemoglobin, the quaternary structural bridge between the α-carboxyl of His-β146 and Lys-α40 is likely to be broken, so the increased negative charge from its carboxyl group could raise the pK of the imidazole by at least as much as 0.4 pH unit (Perrin, 1965). (Note that if the pK_a of His-β146 were raised to a value of 7.8 in methemoglobin then binding of a ligand, such as cyanide, would require a pK lowering of about one unit to 6.7–6.8 since the structure of cyanomethemoglobin is believed to be essentially identical with that of oxyhemoglobin. Such a change would be expected to result in proton uptake on ligand binding to methemoglobin, as has been observed (Anusien *et al.*, 1968) for binding of azide and cyanide.)

If His-β146 is involved in Hb^{TFA}-III's spectrum, the observed titration curve again reflects the changing position of the carboxyl-terminus equilibrium. But in order for His-β146 to form a stable salt bridge to Asp-β94, Tyr-β145 must be able to occupy its bound conformation between the F and G helices. In oxyhemoglobin, conformation changes have occurred which close the F–G pocket and move the carboxy-terminus equilibrium far toward the free position. However, it has been proposed that the trigger for these changes is the movement of the iron atom into the plane of the porphyrin ring (Hoard, 1966; Perutz, 1970b). By analogy with myoglobin derivatives and iron porphyrin models, Perutz has suggested that in deoxyhemoglobin, the iron atom lies 0.75 Å above the heme plane, in oxyhemoglobin it has moved down to within 0.05 Å, of that plane, and in acid methemoglobin, the iron lies 0.3 Å above the plane–slightly more than halfway between the two extremes. This incomplete transition might not drive the equilibrium of the His-β146–Asp-β94 salt bridge as far toward the free position as is the case in oxyhemoglobin.

While it is feasible that His-β146 influences the nmr probe in Hb^{TFA}-III, it certainly is not the only possibility. As already stated a process having a pK_a of 7.8 could be due to a different histidine or to an α-amino group, which, in either case, does not produce the same effect in Hb^{TFA} or Hb^{TFA}-O_2. In order for one of these groups to directly or indirectly affect the environment of Cys-β93 to the extent observed extensive perturbations in the protein must be invoked which would require that Hb^{TFA}-III have a conformation substantially different from either Hb^{TFA} or Hb^{TFA}-O_2 in some pH ranges.

Distinguishing among histidines-97, -143, and -146 as the source of the observed ionization of pK_a = 7.8 in Hb^{TFA}-III is presently not possible on the basis of the nmr evidence. What is clear is that the conformation of the carboxy-terminal region of Hb^{TFA}-III differs from that of Hb^{TFA}-O_2 and possibly also from that of Hb^{TFA}. The importance of interactions in this region to the cooperative processes of ferrous hemoglobin suggests that any differences which exist in methemoglobin are likely to have substantial functional consequences. Hence it is significant that previous investigations (Antonini *et al.*, 1964) have shown that the oxidation of deoxyhemoglobin to methemoglobin is not exactly analogous to the ligand binding process. Oxidation is cooperative, but the Hill coefficient is pH dependent and varies from near 1 below pH 7 to 2.6 above pH 8. Significantly, the midpoint of the transition occurs at about pH 7.4, which suggests the influence of His-β146 on the oxidation process. Further, the Bohr effect for oxidation is larger than that for ligand binding, and part of the increase (the "residual oxidation Bohr effect) cannot be accounted for by the ionization of the water ligand of the ferric heme (Brunori *et al.*, 1969). Any groups in methemoglobin whose pK's differ from those exhibited in deoxy- and oxyhemoglobin could well be involved in these phenomena. Information on these questions is being sought through experiments aimed at identification of the groups responsible for the nmr behavior of Hb^{TFA}-III.

In conclusion, these experiments using fluorine labels have permitted some detailed study of conformational processes in a stretegic region of hemoglobin. It is hoped that these results and the results of experiments now in progress will lead us to a better understanding in molecular terms of the complex regulatory interactions in hemoglobin, the most extensively studied of all allosteric proteins.

References

Antonini, E., Wyman, J., Brunori, M., Taylor, J. F., Rossi-Fanelli, A., and Caputo, A. (1964), *J. Biol. Chem. 239*, 907.

Antonini, E., Wyman, J., Moretti, R., and Rossi-Fanelli, A. (1963), *Biochim. Biophys. Acta 71*, 124.
Anusien, A. C., Beetlestone, J. G., and Irvine, D. H. (1968), *J. Chem. Soc. A*, 960.
Benesch, R., and Benesch, R. E. (1961), *J. Biol. Chem. 236*, 405.
Benesch, R. E., and Benesch, R. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 1101.
Benesch, R. E., Benesch, R., and Yu, C. I. (1969), *Biochemistry 8*, 2567.
Briehl, R. W. (1962), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 21*, 72.
Brunori, E., Taylor, J. F., Antonini, E., and Wyman, J. (1969), *Biochemistry 7*, 2880.
Cohn, E. J., and Edsall, J. T., Ed. (1943), Proteins, Amino Acids, and Peptides, New York, N. Y., Reinhold.
Coryell, C. D., Stitt, F., and Pauling, L. (1937), *J. Amer. Chem. Soc. 59*, 633.
Deal, W. J., Mohlman, S. G., and Sprang, M. L. (1971), *Science 171*, 1147.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Fabry, T. L., Kim, J., Koenig, S. H., and Schillinger, W. W. (1971), Proceedings of the Johnson Foundation Meeting on Heme and Hemoproteins, New York, N. Y., Academic Press.
Garby, L., Gerber, G., and deVerdeer, C. H. (1969), *Eur. J. Biochem. 10*, 110.
George, P., and Hanania, G. (1953), *Biochem. J. 55*, 236.
Haurowitz, F. (1938), *Z. Physiol. Chem. 254*, 266.
Hoard, J. L. (1966), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic Press, p 9.
Kilmartin, J. V., and Wootton, J. F. (1970), *Nature (London) 228*, 766.
McConnell, H., Deal, W. J., and Ogata, R. G. (1969), *Biochemistry 8*, 2580.
Moffat, J. K. (1971), *J. Mol. Biol. 55*, 135.
Moffat, J. K., Simon, S., and Konigsberg, W. (1971), *J. Mol. Biol. 58*, 891.
Muirhead, H., and Greer, J. (1970), *Nature (London) 228*, 516.
Perutz, M. (1970a), *9th Int. Congr. Biochem., Interlaken*, 5.
Perutz, M. (1970b), *Nature (London) 228*, 726.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219*, 131.
Perrin, D. D. (1965), Dissociation Constants of Organic Acids in Aqueous Solution, London, Butterworths.
Riggs, A. (1961), *J. Biol. Chem. 236*, 1948.
Riggs, A., and Wohlbach, R. A. (1956), *J. Gen. Physiol. 39*, 585.
Tomita, S., Enoki, Y., Santa, M., Loshida, H., and Yosuminisu, Y. (1968), *Nara Igaku Zasshi 19*, 1.
Tyuma, I., and Shimizu, K. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 112.
Tyuma, I., Shimizu, K., and Imai, K. (1971), *Biochem. Biophys. Res. Commun. 43*, 423.
Wyman, J., and Allen, D. W. (1951), *J. Polymer Sci. 7*, 449.
Zito, R., Antonini, E., and Wyman, J. (1964), *J. Biol. Chem. 239*, 1809.

Electrofocusing Analysis of HeLa Cell Metaphase Chromosomes†

Aurora M. Landel, Yosef Aloni,‡ Michael A. Raftery,* and Giuseppe Attardi

ABSTRACT: The possibility of fractionating HeLa cell metaphase chromosomes by the electrofocusing technique has been investigated. By applying a modification of the standard procedure, which allows a considerable shortening of the electrofocusing time, these chromosomes could be resolved partially into groups in the pH range from 3.90 to 4.30. These groups, upon reelectrofocusing, banded at the same pH value as in the original run, with improved resolution and an apparent preservation of the morphological integrity of the chromosomes. Electrofocusing of the small HeLa cell chromosomes, previously separated by sedimentation velocity, produced an appreciable enrichment of rRNA genes in subfractions of these chromosomes.

Several reports have been published on the isolation and fractionation of metaphase chromosomes. The materials used in these studies were mammalian cell lines cultivated *in vitro* (Somers *et al.*, 1963; Lin and Chargaff, 1964; Salzman *et al.*, 1966; Huberman and Attardi, 1966, 1967; Mendelsohn *et al.*, 1968; Maio and Schildkraut, 1967; Schneider and Salzman, 1970), mouse ascites tumor cells (Cantor and Hearst, 1966), and mouse leukemia cells (Chorazy *et al.*, 1963). The fractionation procedures utilized were velocity sedimentation in sucrose gradients or in zonal rotors and filtration through porous stainless steel filters. These methods are based on differences in the size and morphology among metaphase chromosomes. In the present work, the applicability of the electrofocusing technique (Vesterberg and Svensson, 1966), which has been used successfully in the separation of macromolecules, to the fractionation of metaphase chromosomes has been investigated.

† Contribution No. 4299 from the Church Laboratory of Chemical Biology and Joint Contribution with the Division of Biology, California Institute of Technology, Pasadena, California 91109. *Received August 8, 1971.* Supported by U. S. Public Health Service Grants GM-14452 to M. A. R. and GM-11726 to G. A. Paper III in the series: Studies of Fractionated HeLa Cell Metaphase Chromosomes. Paper II is by Aloni *et al.* (1971).

‡ Research Fellow of the International Agency for Research on Cancer of the World Health Organization. Present address: Weizmann Institute of Science, Rehovot, Israel.

* Alfred P. Sloan Fellow, 1971; National Institutes of Health Career Development Recipient, 1971.