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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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[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.

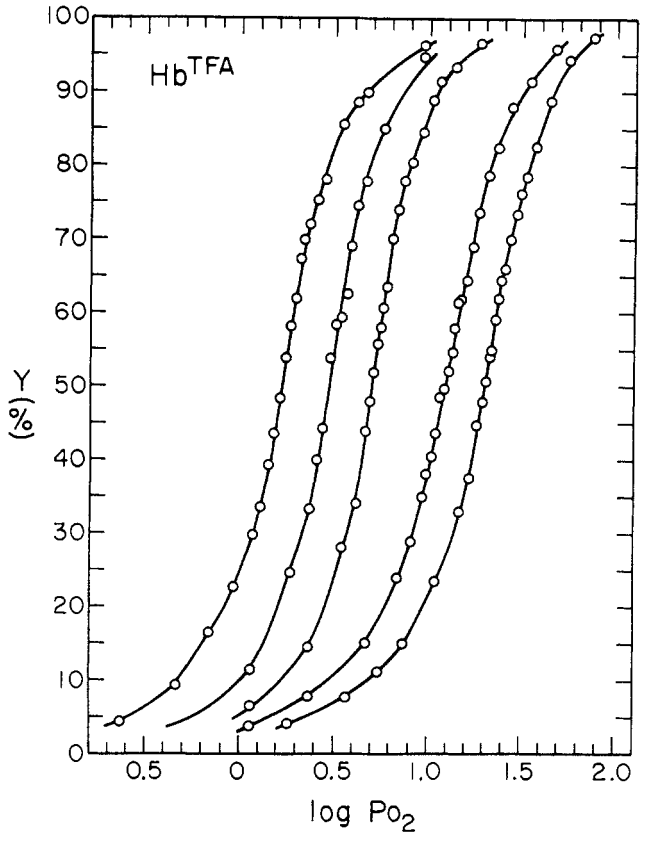

FIGURE 1: Oxygenation curves of Hb^{TFA} at 25°. From left to right: pH 9.0, 8.3, 7.5, 7.0 (in bis-tris + DPG); pH 6.75 (displaced to the right by 0.2 unit for clarity).

For the study of ionic strength effects on oxygenation equilibria, 100 μl of the stock solution (10% in hemoglobin, 0.01 M in bis-tris at pH 7.0) was added to 4.9 ml of buffer solution of the desired ionic strength. DPG solution was then added to the hemoglobin solution in 2:1 molar ratio ($\sim 10^{-4}$ M).

Oxygenation Equilibrium Study. The hemoglobin solution (4 ml) was pipetted into a tonometer and its OD_{540} measured. It was then deoxygenated by repeated washing with nitrogen until its OD_{540} reached a constant value. Oxygenation was carried out by addition of aliquots of air through a serum cap with a syringe, followed by equilibration of the solution by rotating the tonometer for 5 min. OD_{540} of the solution was then measured. Calculation of the oxygen pressure and fractional saturation at each step was carried out as described by Riggs and Wohlbach (1956). Hill coefficients were obtained

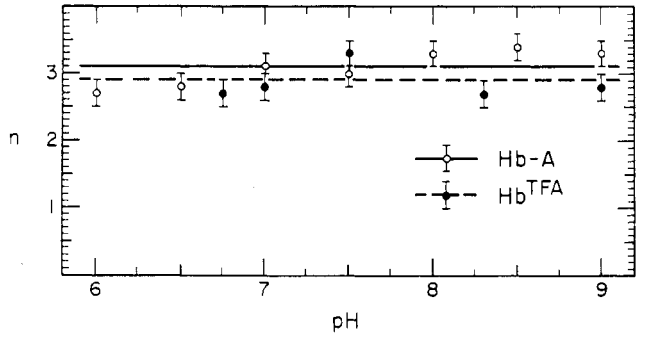

FIGURE 2: Hill coefficient (n) *vs.* pH for Hb-A and Hb^{TFA}.

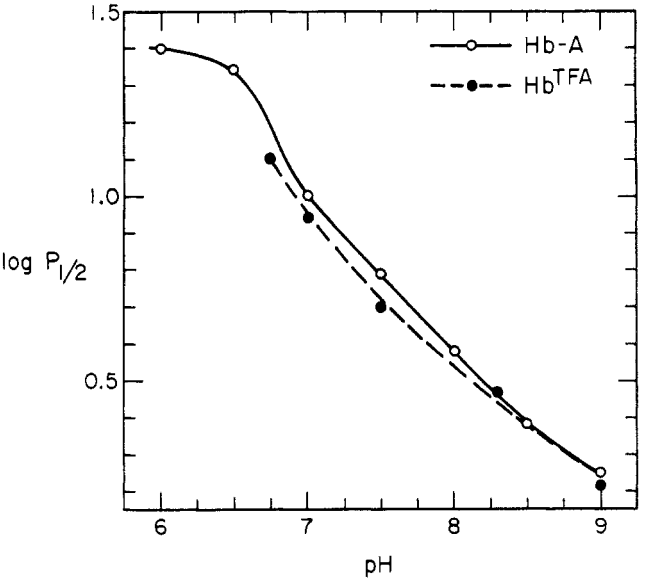

FIGURE 3: Bohr effect for Hb-A and Hb^{TFA}.

from the slopes of log–log plots of oxygen pressure *vs.* $Y/(1 - Y)$, determined by least-squares fits.

Buffer Solutions. pH buffers used were: 0.1 M sodium phosphate solutions at pH's 6, 6.5, and 6.75; 0.1 M sodium phosphate + 0.05 M Tris solutions at pH's 7.5, 8.0 and 8.3; 0.1 M sodium phosphate + 0.05 M sodium borate solutions at pH's 8.5 and 9.0. Buffers used for study of ionic strength effects were 0.1 and 5×10^{-3} M bis-tris at pH 7.0.

DPG Binding Studies. The release of DPG from Hb-A and Hb^{TFA} with increasing fractional ligand saturation was determined by concurrent observation of changes in the visible and ^{31}P nmr spectra of hemoglobin solutions as the carbon monoxide pressure was increased. Visible and nmr spectra were observed concurrently using an nmr tube with a cuvet (1.0-mm path length) fused to the top, as previously described (Huestis and Raftery, 1973). Nmr solutions contained 325 mg of Hb-A or Hb^{TFA} in 2.5 ml of bis-tris–NaCl (pH 7.25, 0.1 M bis-tris–0.1 M NaCl). DPG was introduced as a concentrated solution in a 1.5:1 molar ratio to hemoglobin. EDTA (1 mg) was added to each solution. The sample solution was deoxygenated by repeated washing with nitrogen. Absorbance of the solution at 650 nm was determined before and after each nmr spectrum was recorded. Aliquots of carbon monoxide were introduced by syringe through a rubber septum over the tube opening, and the solution was allowed to equilibrate with the gas mixture before the spectrum was recorded.

Results

Effect of pH on the Oxygenation Equilibria. The oxygenation equilibrium curves for Hb^{TFA} are shown in Figure 1. The Hill coefficients of both proteins were within experimental error invariant with pH in the range 6.0–9.0, with values averaging 3.1 and 2.9 for Hb-A and Hb^{TFA}, respectively (Figure 2). For both Hb-A and Hb^{TFA}, normal Bohr effects were observed (Figure 3). Hb^{TFA} exhibited oxygen affinities slightly higher than those of Hb-A at all pH's studied (*e.g.*, at pH 9, $P_{0.5}$ was 1.7 mm for Hb^{TFA} and 1.8 mm for Hb-A; at pH 7.5, $P_{0.5}$ was 5 mm for Hb^{TFA} and 6.2 mm for Hb-A.

Effect of DPG and Ionic Strength on the Oxygenation Equi-

TABLE I: Allosteric Parameters of Hb-A and Hb^{TFA} at pH 7.0.

	$P_{0.5}$ (mm)	n
Hb-A		
0.1 M bis-tris + 10^{-4} M DPG	21.7	3.1
5×10^{-3} M bis-tris + 10^{-4} M DPG	19.1	2.8
Hb^{TFA}		
0.1 M bis-tris + 10^{-4} M DPG	12.3	2.8
5×10^{-3} M bis-tris + 10^{-4} M DPG	12.9	2.7

libria. Upon addition of DPG at pH 7.0, $P_{0.5}$ was raised from 10.9 to 21.7 mm of oxygen and n increased from 2.7 to 3.1 for Hb-A. For Hb^{TFA}, $P_{0.5}$ and n followed the same trend. In the presence of 10^{-4} M DPG, the ionic strength of the solution could be varied from 5×10^{-3} to 0.1 M without significant change in either oxygen affinity or Hill coefficient for both Hb and Hb^{TFA} (Table I).

Dissociation of DPG from Hb-A and Hb^{TFA} on Ligand Binding. Hb^{TFA} was found to be indistinguishable from Hb-A in the release of DPG as a function of ligand binding. As is shown in Figure 4, DPG dissociation lagged behind overall ligand binding for Hb-A and Hb^{TFA}, and at all fractional saturation values DPG release was identical for both proteins (Figure 5).

Discussion

Oxygen Equilibria of $Hb\text{-}A^{TFA}$. In a recent ^{19}F nmr study of cooperative interactions in hemoglobin (Huestis and Raftery, 1972a), it was found that trifluoroacetonylated hemoglobin exhibited an oxygen affinity almost identical with that of native hemoglobin and a Hill coefficient $n = 2.5$ at pH 7.0 in the absence of DPG.

We have now studied in more detail the fundamental properties of this modified oxygen carrier and our results show clearly that trifluoroacetonylation of Cys-β93 produced very small perturbations of the allosteric properties of the native protein: n was independent of ionic strength and was invariant with pH for either Hb-A or Hb^{TFA} with average values of 3.1 and 2.9, respectively, 0.1 M phosphate (Figure 2); furthermore, Bohr effects of Hb-A and Hb^{TFA} were almost identical (Figure 3).

DPG Binding to Hb-A and Hb^{TFA}.The use of ^{31}P nmr to study dissociation of DPG from hemoglobin on carboxygenation has been described previously (Huestis and Raftery, 1972d). In that communication, the fraction of DPG bound to Hb-A (determined from the chemical shifts of the phosphate resonances) was compared to the fractional ligand binding (determined from the fractional change in the visible spectrum) at that ligand pressure. It was found that release of DPG from deoxyhemoglobin lagged behind the ligand binding (Figure 4a). The same experiment, repeated with Hb^{TFA}, showed an identical lag (Figure 4b). As is shown in Figure 5, the fraction of bound DPG at a given ligand saturation is the same for Hb-A and Hb^{TFA}

Therefore it is possible to compare ^{31}P nmr data on the release of DPG with ^{19}F nmr data on conformation changes in the β chains occurring on ligand binding. In previous work (Huestis and Raftery, 1972c), the ^{19}F nmr spectrum of Hb^{TFA} has been used to monitor the populations of hemoglobin species containing none, two, three, and four ligand molecules. Figure 6 summarizes these findings; the fractional populations of liganded species are shown as functions of the ligand saturation. Comparison of these populations to the fraction of bound DPG at corresponding values of Y yields information on the stage of ligand binding at which DPG dissociates. As was found for Hb-A (Huestis and Raftery, 1972d), release of DPG from Hb^{TFA} precedes the production of the four-liganded species $Hb(CO)_4$, but lags behind the production of the sum of $Hb(CO)_4$ and $Hb(CO)_3$ (Figure 7). This indicates that for Hb^{TFA}, as for Hb-A, DPG does not dissociate cleanly at a single stage of ligation, but appears to bind partially to the three liganded species. (The species containing three ligands has been identified as $\alpha_2^{CO}\beta^{CO}\beta^{deO_2}$ in Huestis and Raftery (1972c)). Hence the fraction of liganded β chains, Y_β, is equal to the sum of fractional populations of $Hb(CO)_4$ plus half of $Hb(CO)_3$. As is shown in Figure 7, the release of DPG is exactly proportional to Y_β.)

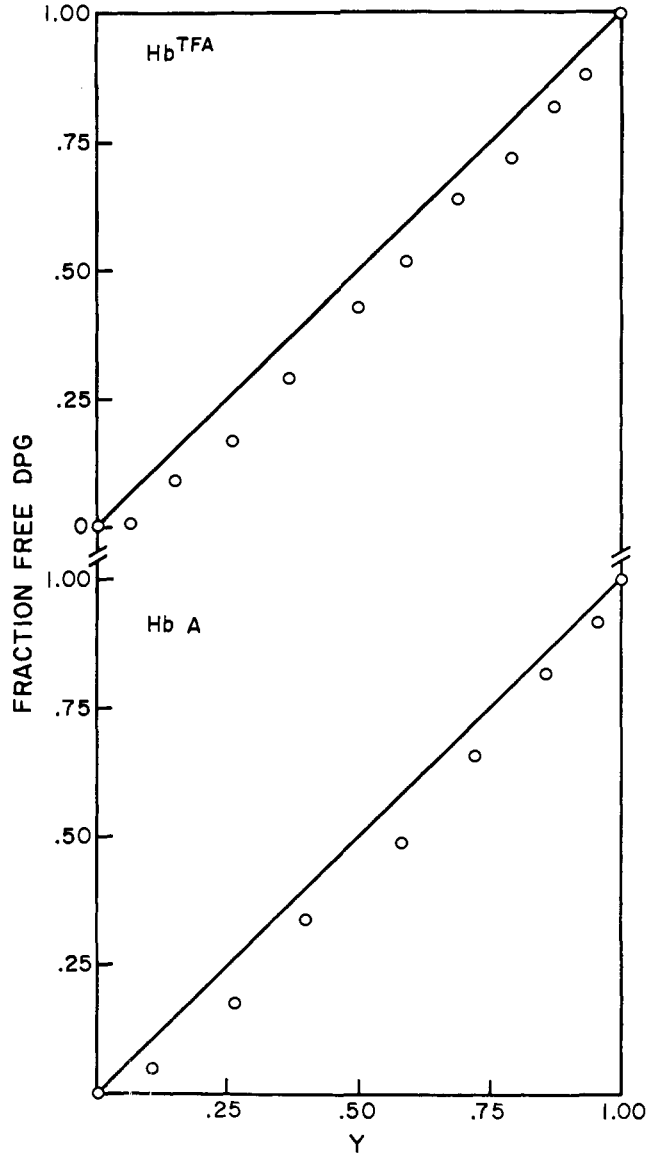

FIGURE 4: The fraction of DPG which has dissociated from Hb-A and Hb^{TFA}, as a function of the fraction of bound carbon monoxide.

Studies of the oxygen binding equilibrium of Hb^{TFA} and its response to pH changes and organic phosphates thus indicate that Hb^{TFA} is very similar to native hemoglobin in all of its functions. The principal difference is a decline in the Hill coefficient from 3.1 to 2.9. This remaining relatively high level of

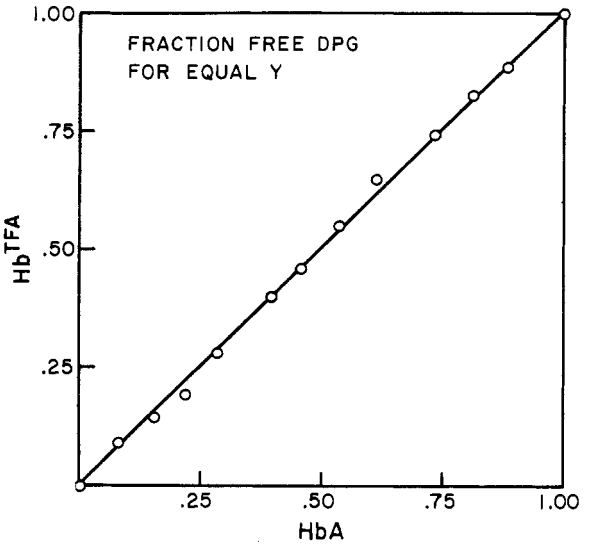

FIGURE 5: The fraction of DPG released from Hb-A *vs.* the fraction released from Hb^{TFA} at the same values of Y.

cooperativity, plus the similarities in oxygen affinity, Bohr effect, and DPG binding, indicate that the ^{19}F nmr probe in Hb^{TFA} reflects natural conformational processes. Thus Hb^{TFA} can be used for significant investigations of the properties of hemoglobin.

pH and Salt Effects on the Oxygenation Equilibrium. In the course of these studies comparing functional properties of Hb-A and Hb^{TFA}, ionic strength and pH effects were examined. It was found that if the DPG binding effect (*e.g.*, lowered ligand affinity and slightly increased cooperativity) was properly controlled, pH and salt concentration did not affect the cooperativity. The contradictory literature on these effects (Antonini *et al.*, 1962; Kilmartin and Hewitt, 1971; Benesch *et al.*, 1969) is due primarily to incomplete saturation of the DPG binding site at high pH's and low ionic strengths. Antonini reported decreased Hill coefficients at very low

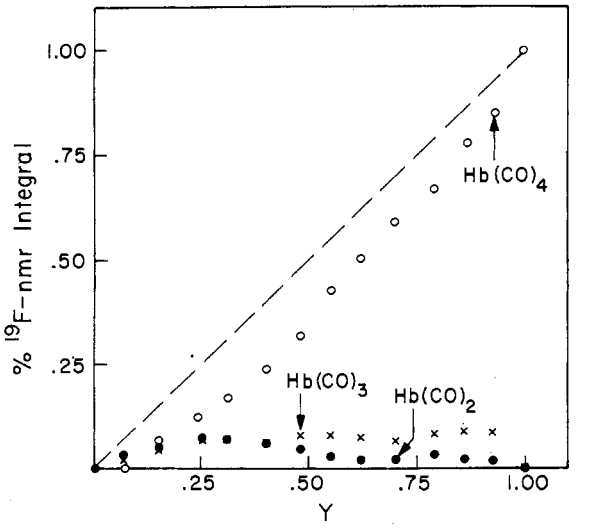

FIGURE 6: The fractional populations of two-, three-, and four-liganded tetramers of Hb^{TFA}, as functions of carbon monoxide saturation. (Data from Huestis and Raftery, 1973). It should be noted that several points, including those at $Y = 1.0$, overlap for the doubly and triply liganded species.

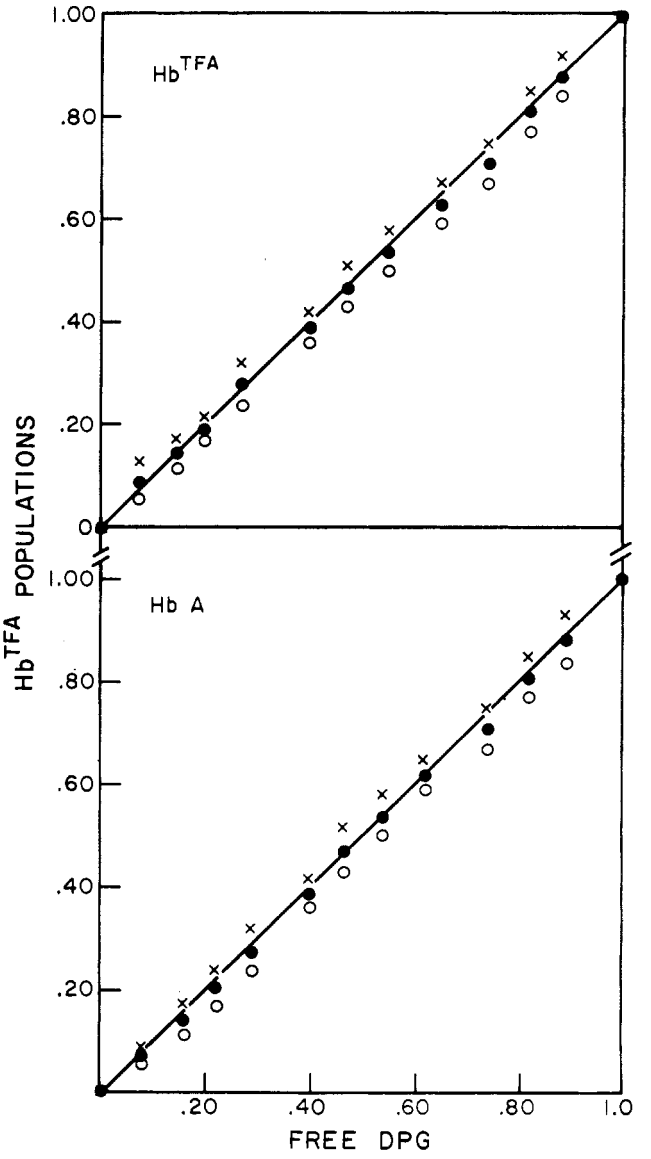

FIGURE 7: Comparison of fractional DPG release to the populations of liganded peaks for Hb-A and Hb^{TFA}: (×) $Hb(CO)_4$, (●) $Hb(CO)_4 + {}^1/_2\ Hb(CO)_3$ (Y_β), and (○) $Hb(CO)_4 + Hb(CO)_3$.

($<10^{-2}$ M) salt concentrations, but Benesch and Benesch have shown that low ionic strengths coupled with partial removal of DPG produce spurious flattened binding curves due to binding to a mixture of DPG-stabilized deoxyhemoglobin (which has low ligand affinity) and "stripped" (high affinity) deoxyhemoglobin. The Beneschs' work showed that the two curves contributing to the flattened composite were normal. Our findings substantiate this result at very low concentrations ($\sim 5 \times 10^{-3}$ M), where Hill coefficients of 2.8 and 2.7 were obtained for Hb-A and Hb^{TFA} in the presence of 10^{-4} M DPG.

Similarly, Kilmartin and Hewitt's (1971) observation of diminished cooperativity at pH's above 8 in 0.05 M borate is probably due to incomplete binding of borate anions to the DPG site, with resulting heterogeneity of the deoxyhemoglobin. DPG binding is much weaker at high pH, but use of 0.1 M phosphate in addition to the borate buffer yielded normal Hill coefficients for Hb-A and Hb^{TFA} up to pH 9 (Figure 2).

Thus, in studies in which cooperative ligand binding is separated from the DPG effect, the cooperative process was

shown to be insensitive to the pH and salt concentration of the medium. This separation of effects allows us to examine the forces involved in cooperative conformation transitions in regions of the hemoglobin molecule other than the DPG binding site. A recent proposal (Perutz, 1970) suggested that the cooperative transition was governed by ionic interactions between various charged groups in deoxyhemoglobin, which attractions are disrupted by the change to the liganded form exposing the charged groups to the solvent. The Bohr effect and DPG effects are demonstrably due to this kind of electrostatic forces, and, in contrast to the Hill coefficient, the Bohr and DPG effects are influenced strongly by the ionic strength of the medium (Antonini *et al.*, 1962). At high salt concentrations, the Bohr effect is diminished and the DPG effect overwhelmed, as might be expected from increased stabilization of the exposed state of the pertinent charged residues. Similarly, if charged groups such as histidine-β146 and arginine-α141 are involved in energetically important associations in deoxyhemoglobin and move freely in the solvent in oxyhemoglobin, high ionic strengths should stabilize the oxy (exposed) form, producing increased ligand affinity. In fact, at very high ionic strengths the ligand affinity decreases (Benesch *et al.*, 1969), indicating stabilization of the deoxy form even apart from the DPG effect. It seems likely from this result that the burying of hydrophobic groups such as tyrosine-β145 in nonpolar regions of the protein may be an important factor in stabilization of the deoxy form, overriding the contributions of the salt bridges.

In addition to being insensitive to ionic strength, the Hill coefficient has been shown to be independent of pH. The importance of the histidine-β146 salt bridges to stabilization of the deoxy form is thus called into question, since previous work (Huestis and Raftery, 1972b) has shown that histidine-β146 is deprotonated and hence uncharged above pH 8.

Summary

The allosteric properties of the trifluoroacetonylated derivative Hb^{TFA} have been compared to those of native Hb-A. The Bohr effect, oxygen affinity, and DPG effect were shown to be very similar, and the cooperativity was found to be very slightly decreased (n average = 2.9). Hb^{TFA} is thus a useful derivative for study of conformation processes in hemoglobin by ^{19}F nmr. In the course of the comparative studies, the binding of DPG to partially liganded tetramers was studied, and results from Bohr effect and ionic strength studies yielded insight into the forces contributing to the cooperative conformation transition.

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