Hydrogen–Tritium Exchange Survey of Allosteric Effects in Hemoglobin†

Joan J. Englander and S. Walter Englander*
Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Received September 23, 1986; Revised Manuscript Received December 12, 1986

ABSTRACT: The oxy and deoxy forms of hemoglobin display major differences in H-exchange behavior. Hydrogen–tritium exchange experiments on hemoglobin were performed in the low-resolution mode to observe the dependence of these differences on pH (Bohr effect), organic phosphates, and salt. Unlike a prior report, increasing pH was found to decrease the oxy–deoxy difference monotonically, in general accordance with the alkaline Bohr effect. A prior report that the H-exchange difference between oxy- and deoxyhemoglobin vanishes at pH 9, and thus appears to reflect the Bohr effect alone, was found to be due to the borate buffer used, which at high pH tends to abolish the oxy–deoxy difference in a limited region of the H-exchange curve. Effects on hemoglobin H exchange due to organic phosphates parallel the differential binding of these agents (inositol hexaphosphate more than diphosphoglycerate, deoxy more than oxy, at low pH more than at high pH). Added salt slows H exchange of deoxyhemoglobin and has no effect on the oxy form. These results display the sensitivity of simple H-exchange measurements for finding and characterizing effects on structure and dynamics that may occur anywhere in the protein and help to define conditions for higher resolution approaches that can localize the changes observed.

Hemoglobin carries hundreds of hydrogens that exchange with solvent protons over an exceedingly wide range of rates. The exchange rate of each individual hydrogen depends upon its involvement in structure and details of protein structural dynamics in its immediate vicinity. Thus, observation of the nonperturbing H-exchange process can *in principle* provide information about structure, structure change, structural fluctuations, and the interplay of these with function at multiple probe points throughout the protein.

In practice this capability is limited by problems of measurement. High-resolution methods are available, namely, two-dimensional ^{1}H NMR and neutron diffraction, which can in favorable cases monitor the exchange of individual protons and thus localize functionally interesting changes in a protein, but these approaches have not yet proven applicable to the study of hemoglobin H exchange. The survey described here was intended in part to test the ability of hydrogen-exchange measurements done in the simplest low-resolution mode, with the tritium–gel filtration method (Englander & Englander, 1978), to detect functionally interesting changes in hemoglobin. If a change can be detected in this way, then the same methods can be used to characterize that change in some detail. These experiments further serve to define the conditions for more advanced medium-resolution experiments that can be used to selectively tag with tritium just the protein segments that change and localize them within the protein (Rosa & Richards, 1979, 1981; Lennick & Allewell, 1981; Burz & Allewell, 1986; Englander et al., 1983; Ray & Englander, 1986).

The literature contains a quantity of hydrogen-exchange (HX)[1] data for various proteins measured in the low-resolution mode [see Hvidt and Nielsen (1964), Barksdale and Rosenberg (1982), Woodward et al. (1982), and Englander and Kallenbach (1984)]. In the case of hemoglobin the data are fragmentary and in some cases contradictory. In this work, H exchange measured in this way was used to compare the exchange curves of oxy- and deoxyhemoglobin under a variety of functionally interesting conditions. Various effectors of the allosteric transition were studied, namely, pH (the Bohr effect), organic phosphates, and salt. In addition, a specific effect of added borate on hemoglobin was recognized. The results show that these kinds of experiments can detect the occurrence of structural effects in hemoglobin with high sensitivity and, more specifically, help to clarify a number of observations in the literature.

Materials and Methods

Sample Preparation. Hemoglobin was prepared from freshly drawn human blood. Red cells were centrifugally washed and pelleted, lysed by osmotic shock (3 volumes of deionized water), and spun to remove stroma (after addition of 0.1 M NaCl). Hemoglobin was stripped of diphosphoglycerate by passage through a Sephadex column (Berman et al, 1971; residual DPG measured by a coupled enzyme reaction with Sigma kit 35-UV).

Bistris buffer at 0.05 M was used for HX experiments at pH 6.6 and 7.4 and Tris at pH 8.2 and 8.8, except as otherwise noted. pH in the buffer solutions was set at room temperature to provide the desired pH at the experimental temperature ($\Delta H°$ is 9 kcal for Tris and 7 kcal for Bistris; S. Gill, personal communication).

Tritium Exchange. For initial labeling of hemoglobin by inexchange in tritiated water, samples were first dialyzed into 0.05 M Bistris buffer at pH 7.5 and then incubated in the presence of 2 mCi/mL tritiated water at 20 °C for 3 days. This condition does not achieve complete labeling (the slowest ~15% of peptide NH escape labeling), but was chosen to minimize oxidation to methemoglobin, which is promoted at the higher pH and temperature required to produce faster, more complete labeling. In fact, complete labeling is, for most studies, undesirable (see below).

Exchange-out of bound tritium was initiated by passage of hemoglobin samples (0.3 mL) through a small Sephadex column (1 × 6 cm, G-25 fine) to remove free tritium. This run also served to reset the solvent to the desired pH and salt

† This work was supported by NIH Research Grant AM 11295.

[1] Abbreviations: HX, hydrogen exchange; Hb, hemoglobin; DPG, diphosphoglycerate; IHP, inositol hexaphosphate; Tris, tris(hydroxymethyl)aminomethane; Bistris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/87/0426-1846$01.50/0

condition. [For experiments with diphophoglycerate (DPG), the column was not filled with DPG solution; rather, DPG was added to the sample just before the Sephadex run and again to the effluent, exchange-out pool.] The hemoglobin was then allowed to exchange-out in the chosen solvent condition at 0 or 22 °C. Aliquots were taken in time and passed through a second Sephadex column to remove solvent tritium, and the eluant hemoglobin was measured to determine remaining unexchanged H per hemoglobin dimer. The second Sephadex columns were always at 0 °C.

H remaining unexchanged was calculated for samples obtained from the second Sephadex column run. For each run, several samples (~2 drops each) taken through the effluent protein peak were diluted to about 1 mL by addition of a modified Drabkin's solution, and protein concentration in the cyanomet form was measured in each sample by absorbance at 420 nm. Tritium remaining bound was then measured by pipeting 0.5 mL of each sample into liquid scintillation counting vials. Background absorbance and counts were measured in prepeak samples. From these parameters one can calculate the number of sites remaining unexchanged per hemoglobin molecule as a function of exchange-out time. For further details and discussion of methodology, see Englander and Englander (1978).

Deoxygenation. For exchange-out in the deoxy form, hemoglobin samples at 0 °C were deoxygenated by addition of a few crystals of $Na_2S_2O_4$ (dithionite) and passed immediately through a first Sephadex column. This column had been previously cleared of oxygen by washing with deoxygenated buffer containing (in addition to the intended experimental solute levels) 0.3% D-glucose, 30 μg/mL glucose oxidase, 6 μg/mL catalase, and 0.1 mM EDTA. The deoxyhemoglobin exchange-out pool was collected directly into and exchanged-out in a stoppered test tube under flowing argon gas washed through an inorganic oxygen-scavenging system (Meites & Meites, 1965).

Methemoglobin Content. At the conclusion of each set of deoxy exchange-out runs, the remaining exchange-out pool (first column effluent) was checked for methemoglobin content. A small sample was diluted into CO-equilibrated buffer and its optical density (OD) measured at 418 nm before and after addition of a few crystals of dithionite. In dithionite, the methemoglobin present becomes reduced and binds CO. Fraction methemoglobin is 1.38 × ΔOD/final OD. When more than 4% of methemoglobin was present at day's end, the entire data set was discarded. It is important to note that methemoglobin content tends to increase during the experimental exchange-out time (several hours), and this is more pronounced at pH values away from pH 7.4, both lower and higher. Methemoglobin formation is also promoted at low salt (0.05 M Tris) and high salt (0.5 M NaCl) and at higher temperature, and its formation is autocatalytic when significant amounts are initially present.

RESULTS

Figure 1 shows H-exchange curves for oxy- and deoxyhemoglobin. As is well-known, a number of hydrogens exchange more slowly in T-state deoxyhemoglobin than in R-state forms. The measurements in Figure 1 were performed over a range of pH and at two different temperatures in order to scan a significant fraction of the overall hemoglobin H-exchange curve, which extends over many orders of magnitude on the time axis. The stepwise increase in pH and temperature brings slower and slower sections of the overall exchange curve onto a convenient time scale. Since peptide NH exchange is catalyzed by hydroxide ion, one can expect roughly a 10-fold

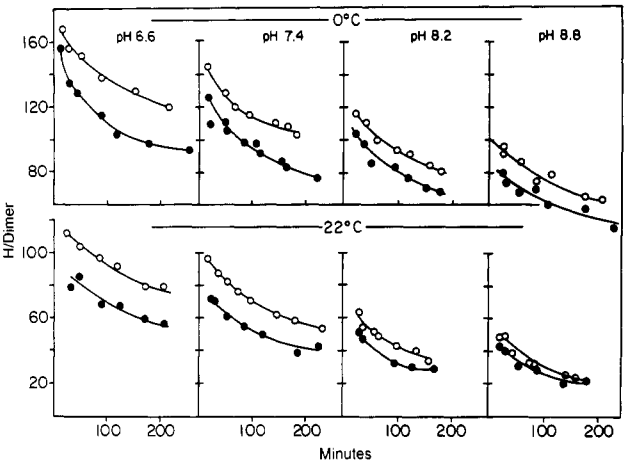

FIGURE 1: Hydrogen–tritium exchange-out curves for hemoglobin in deoxy (○) and oxy (●) forms. Buffers were Bistris at pH 6.6 and 7.4 and Tris at pH 8.2 and 8.9 plus 0.1 M NaCl in all cases. Increasing pH and temperature speed the chemical exchange rate, thus bring slower and slower parts of the exchange curve into the measured time window.

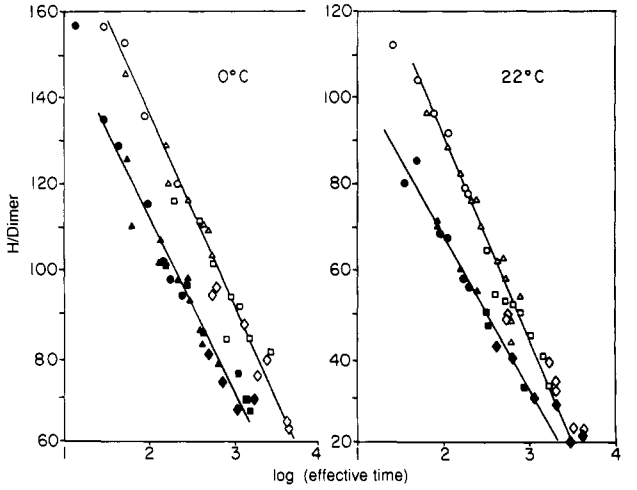

FIGURE 2: Willumsen plot of the data in Figure 1. Data taken at various pH are transformed to the pH 6.6 time scale by use of an empirical pH to time conversion (d[log t]/d[pH] = 0.65), and the extended time scale that results is plotted logarithmically (minutes). The difference between oxyHb (filled symbols) and deoxyHb (open symbols) decreases monotonically with increasing pH. (○, ●) pH 6.6; (△, ▲) pH 7.4; (□, ■) pH 8.2; (◇, ◆) pH 8.8.

increase in rate upon raising the pH one unit, that is, d(log rate)/d(pH) ~ 1. In practice, the pH-dependent increase in rate is somewhat less than 10-fold per pH unit (ca. 5-fold for hemoglobin) owing to the countereffect of the pH-dependent increase in protein-bound anionic charge. Following Willumsen (1971), the data taken over a range of pH (Figure 1) are combined in Figure 2 by plotting the time axis on a logarithmic scale and placing the data taken at various pH onto a common time scale, specifically the time scale that obtains at pH 6.6, by applying the empirical conversion factor d(log t)/d(pH) 0.65.

pH Effects. The results in Figures 1 and 2 provide information on the overall oxy–deoxy difference as a function of pH. The oxy–deoxy difference changes smoothly with pH, decreasing monotonically as pH increases, both at 0 and at 22 °C. This apparently reflects the alkaline Bohr effect, which is well-known to reduce the differences between oxy- and deoxyhemoglobin as pH is raised through the range studied.

A Specific Borate Effect. In prior work, Hallaway et al. (1984) observed no difference between oxy- and deoxyhemoglobin H exchange at pH 9 (0.05 M borate buffer at 6

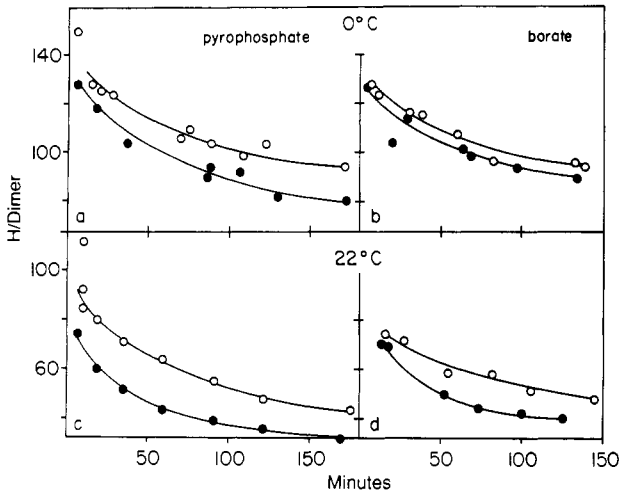

FIGURE 3: Specific effect of borate on hemoglobin H exchange. HX data were taken at pH 9 in 0.01 M pyrophosphate or borate buffer. The presence of borate reduces the oxy–deoxy difference in a limited portion of the overall HX curve (panel b). Compare also Figures 1 and 4 at pH 8.8 and 0 °C in Tris buffer (see notes in text).

°C). This led to their important conclusions concerning the correlation of HX results solely with the alkaline Bohr effect rather than with more general differences between the T and R states. The data in Figure 3b, obtained under the conditions used by Hallaway et al. (except at 0 °C instead of 6 °C), essentially repeat their result (a small difference still appears). As it turns out, the apparently extreme Bohr-dependent effect on hemoglobin H exchange suggested in Figure 3b is misleading.

Measurements under the same solution conditions (pH 9, 0.05 M borate) but at 22 °C, where a lower part of the H-exchange curve is seen (Figure 3d), exhibit a substantial oxy–deoxy difference. Identical experiments at both 0 and 22 °C, but with the borate replaced by pyrophosphate (Figure 3a,b), show a large oxy–deoxy difference. Other measurements under nearly identical conditions (0 °C, pH 8.8) but with the borate buffer replaced by Tris plus 0.1 M NaCl are shown in Figure 1; a sizeable oxy–deoxy difference is found. Substantial oxy–deoxy differences are seen in the high-pH data of Figure 4a (Tris buffer with DPG) and Figure 4b (Tris buffer with IHP).

It appears then that the apparent reduction of the oxy–deoxy difference in Figure 3b depends not only upon the high-pH titration of the Bohr groups but also on the presence of borate buffer and even then is limited to a part of the overall HX curve. Thus, while the HX differences between oxy- and deoxyhemoglobin are reduced at high pH (Figures 1 and 2), they are not solely a marker of the alkaline Bohr effect as suggested by Hallaway et al. (1984).

It is interesting to ask whether the effect of borate seen in Figure 3b represents a lowering of the deoxy curve (acceleration of deoxyHb HX) or a raising of the oxy curve (slowing of oxyHb HX). Direct comparison with the other curves shown here is not possible since, in reproducing the conditions of Hallaway et al. (1984) to obtain Figure 3b, we used more rigorous exchange-in conditions than for the other experiments reported here, so that more very slowly exchanging sites became labeled and the H-exchange curves fell higher on the ordinate. Panels a and c of Figure 3 were also obtained with these same exchange-in conditions but with the borate buffer replaced by pyrophosphate during exchange-out. A comparison shows that, at both 0 and 22 °C, the major effect of borate is to selectively raise the oxy curve (slower oxyHb exchange).

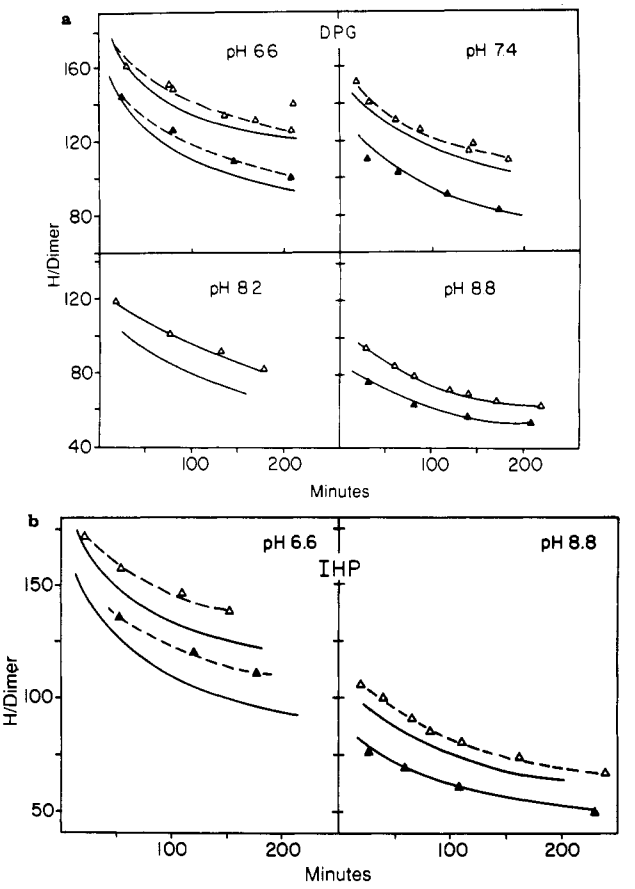

FIGURE 4: Effects of added organic phosphates measured as a function of pH. The solid curves are taken from Figure 1 (DPG-stripped hemoglobin in 0.05 M Tris or Bistris buffer plus 0.1 M NaCl). Data symbols and the dashed curves drawn through them show the H-exchange of deoxy- (△) and oxy- (▲) hemoglobin in the presence of DPG at 2.5 mM or IHP at 1 mM. HX slowing due to the added phosphates parallels their binding behavior.

Organic Phosphates. The influence on hemoglobin H exchange of the allosteric effector diphosphoglycerate (DPG) is shown in Figure 4a, and some results for the stronger effector inositol hexaphosphate (IHP) are in Figure 4b (all at 0 °C). The solid curves in this figure are redrawn from Figure 1 to provide a comparison with data measured in the absence of organic phosphates (in 0.05 M Tris or Bistris buffer plus 0.1 M NaCl).

The results follow, at least qualitatively, the binding behavior of the organic phosphates to oxy- and deoxyhemoglobin. Figure 4a shows that DPG (2.5 mM) slows oxyhemoglobin H exchange at pH 6.6 but not at pH 7.4 and higher. DPG slows deoxyhemoglobin exchange at pH 6.6 and 7.4 but not at pH 8.2 and higher. Similarly, 1 mM IHP (Figure 3b) slows exchange in both oxy- and deoxyhemoglobin at low pH (pH 6.6), but at higher pH (pH 8.8) the effect is seen only for the deoxy form.

Salt Effects. Some results indicating the effect of salts on oxy- and deoxyhemoglobin H exchange at pH 7.4 are shown in Figure 5 at 0 and 22 °C. Deoxyhemoglobin H exchange displays small but measurable sensitivity to salt levels. Compared to the solid curve drawn (from Figure 1 at 0.1 M NaCl and 0.05 M Bistris buffer), exchange is slightly faster when the NaCl is omitted (0.05 M Bistris buffer only) and is detectably slowed when the Bistris buffer is replaced by 0.1 M inorganic phosphate. This is not due to a general salt effect on hemoglobin H exchange; under the same conditions, oxyhemoglobin H exchange is unaltered (lower curves in Figure 5).

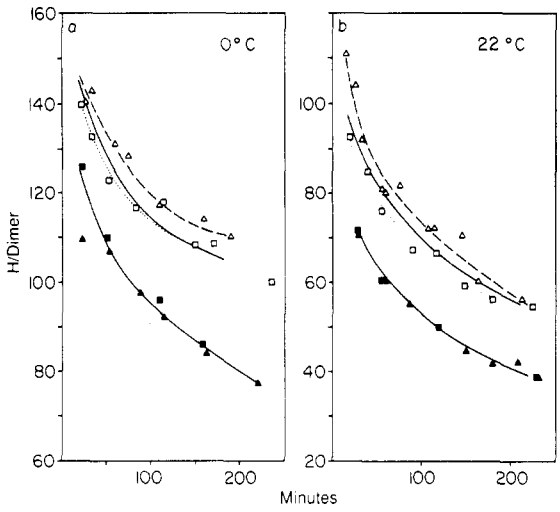

FIGURE 5: Effect of added salts on hemoglobin H exchange at pH 7.4. The solid curves are from Figure 1 (DPG-stripped hemoglobin in 0.05 M Bistris and 0.1 M NaCl). OxyHb (lower curve, filled symbols) is unaffected by salt through the range tested. DeoxyHb (upper curves, open symbols) is slowed by increasing salt. Salt conditions were as follows: [(□, ■) and dotted curve] 0.05 M Bistris, 5 mM Cl^-; (solid curves) 0.05 M Bistris, 0.1 M NaCl (from Figure 1); [(△, ▲) and broken curve] 0.1 M sodium phosphate, 0.1 M NaCl.

Experiments at much higher salt produced serious amounts of methemoglobin and were not pursued. No attempt was made here to delineate specific ion effects (e.g., chloride and phosphate).

Discussion

Major differences between oxy- and deoxyhemoglobin are apparent over the entire hemoglobin H-exchange curve. The HX results described, taken in the low-resolution mode, display modifications of this difference due to pH, phosphates, and salt. Evidently, these methods can detect effects directly in solution that may occur anywhere within the protein and can be used to characterize their behavior. Here we briefly discuss the results obtained in this survey.

pH Effects. The H-exchange difference between deoxyhemoglobin and liganded hemoglobin, displayed in Figure 1, is striking and has been observed before by a number of workers (Englander & Mauel, 1972; Benson et al., 1973; Abaturov et al., 1977; Hedlund et al., 1978). The present data outline the pH dependence of this difference, which is seen to decrease smoothly with increasing pH. The energy gap separating T- and R-state hemoglobin decreases monotonically with increasing pH (Antonini et al., 1965; Chu et al., 1984), and the HX results appear to reflect that trend.

In earlier work Hedlund et al. (1978) reported that the H-exchange oxy–deoxy difference, measured as above, peaks sharply at pH ~7.4 and decreases to small values when the pH is changed up to 8 or down to 7 and below. From this, conclusions were drawn concerning pH-dependent structural rearrangements in both liganded hemoglobin and deoxyhemoglobin between pH 7 and pH 8. We do not confirm these results.

A possible explanation for the divergence between our data and those of Hedlund and co-workers is suggested by the high levels of methemoglobin encountered in the earlier work. Hedlund et al. placed considerable importance on achieving complete tritium in-exchange and therefore subjected their oxyhemoglobin samples to fairly rigorous exchange-in conditions, incubating in tritiated water at elevated pH and temperature for long times (pH 9.8, 32 °C, 24 h, 0.5 mM tetramer), which produced oxidation levels of 12–18%. Subsequent experimental steps (deoxygenation by N_2 flushing, 6-h HX incubations at various pH) may be expected to further increase oxidation levels. These workers attempted to correct for effects due to methemoglobin present by applying calculated factors to the HX data, but in truth, one does not know how to do this.

It is worth noting that in most tritium-exchange experiments full tritium labeling is unnecessary and often undesirable. In measurements of faster exchanging protons, label on slower sites merely acts to produce a higher background and increased noise. The value of previously described selective labeling approaches, kinetic labeling (Calhoun & Englander, 1985) and functional labeling (Englander & Englander, 1983), is that they avoid labeling slower sites and place tritium label preferentially on sites that exchange in the limited time region one wants to target for measurement.

Borate Effect. Results described here show that, although the HX difference between oxy- and deoxyhemoglobin is decreased as pH increases, the difference does not disappear at ph 9, where the alkaline Bohr effect has essentially run its course. A prior report to the contrary appears to reflect a specific effect of the borate anion on a limited part of the HX curve. The ability of borate to interact with protein side chains like arginine has been noted before (Patthy & Smith, 1975), though its binding to hemoglobin has not to our knowledge been studied.

The limited data available support the suggestion of Hallaway et al. (1984) that this effect, seen with borate and also upon cabamylation, may operate preferentially to slow oxyhemoglobin H exchange and move the oxy curve upward on the HX ordinate toward the deoxy curve. It is, however, important to note that this effect is not general. Much previous experimentation shows that when hemoglobin is modified in a way that disfavors the T state, the effect operates always to make deoxyhemoglobin H exchange faster so that the deoxy HX curve is displaced downward toward the liganded curve. For example, Ray and Englander (1986) found this in a study of some allosterically sensitive hydrogens at the α-chain N-terminus, the region of the protein at issue in the considerations of Hallaway et al. (1984). Analogous effects were found for other allosterically sensitive hydrogens by Liem et al. (1980) and Englander et al. (1983). All these observations are fully consistent with the severing of interactions that make the T-state structure the more stable, lower energy protein form (Perutz et al., 1970). While the reverse behavior may appear in the case of borate, one notes that here the allosteric effect is not disabled. The oxygen binding curve of hemoglobin at pH 9 and 20 °C in 0.05 M borate and 0.1 M Cl^- is the same as that measured without borate (Dr. Ruth Benesch, personal communication).

Organic Phosphates. Though the literature records fairly wide disagreement on many of the particulars of organic phosphate binding to hemoglobin, certain general trends are well-known [see Powers et al. (1981) for a review]. At the lowest pH used here (pH 6.6), DPG and IHP bind to both oxy- and deoxyhemoglobin. As the pH is increased, binding decreases for both forms, and oxyhemoglobin loses its binding affinity before deoxyhemoglobin. Throughout, IHP maintains a greater binding affinity than DPG. All these effects are apparent in the HX results (Figure 4).

The phosphate polyanions studied here bind to a defined site in hemoglobin and preferentially stabilize the (slow-exchanging) T conformation. Thus they may slow H-exchange rates in a local sense, immediately about the binding site, and/or might affect more distant allosterically involved sites.

The former mode probably explains the observed slowing of exchange even in oxyhemoglobin; the multisite binding of a polyanion can be expected to act as a cross-linker, stabilizing against the exchange of protons immediately around the binding site. It remains to be seen whether or not longer range effects occur in deoxyhemoglobin. The results obtained suggest that the effects are qualitatively the same in the oxy and deoxy forms and depend only upon the fact of binding.

It can be expected that experiments like these, performed in a higher resolution mode to identify the affected protons [e.g., Ray and Englander (1986)], could directly recognize the binding sites and perhaps other affected, allosterically involved regions, though the present low-resolution experiments give no such information. It is also interesting to note that rather simple HX measurements like those shown here might be used to measure phosphate binding.

ACKNOWLEDGMENTS

We are indebted to Prof. Ruth Benesch for measurements of the hemoglobin oxygen binding curve at pH 9 in the presence of borate buffer.

REFERENCES

Abaturov, L. V., Yakobashvili, N. N., Jinoria, K. S., Molchanova, T. P., & Varshavsky, Y. M. (1977) *FEBS Lett. 77*, 103–106.

Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronticelli, C., & Rossi-Fanelli, A. (1965) *J. Biol. Chem. 240*, 1096.

Barksdale, A. D., & Rosenberg, A. (1982) *Methods Biochem. Anal. 28*, 1–113.

Benson, E. S., Rossi-Fanelli, M. R., Giacomette, G., M., Rosenberg, A., & Antonini, E. (1973) *Biochemistry 12*, 2699–2706.

Berman, M., Benesch, R., & Benesch, R. E. (1971) *Arch. Biochem. Biophys. 145*, 236–239.

Burz, D. S., & Allewell, N. M. (1986) *Biophys. J. 49*, 70–72.

Calhoun, D. B., & Englander, S. W. (1985) *Biochemistry 24*, 2095–2100.

Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry 23*, 604–617.

Englander, J. J., & Englander, S. W. (1978) *Methods Enzymol. 49F*, 24–39.

Englander, J. J., Rogero, J., & Englander, S. W. (1983) *J. Mol. Biol. 169*, 325–344.

Englander, S. W., & Englander, J. J. (1983) in *Structure and Dynamics: Nucleic Acids and Proteins* (Clementi, E., & Sarma, R. H., Eds.) pp 421–433, Adenine, New York.

Englander, S. W., & Kallenbach, N. R. (1984) *Q. Rev. Biophys. 16*, 521–625.

Hallaway, P. E., Hallaway, B. E., & Rosenberg, A. (1984) *Biochemistry 23*, 266–273.

Hedlund, B. E., Hallaway, P. E., Hallaway, B. E., Benson, E. S., & Rosenberg, A. (1978) *J. Biol. Chem. 253*, 3702–3707.

Hvidt, A., & Nielsen, S. O. (1964) *Adv. Protein Chem. 21*, 287–386.

Lennick, M., & Allewell, N. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7055–7059.

Liem, R. K. H., Calhoun, D. B., Englander, J. J., & Englander, S. W. (1980) *J. Biol. Chem. 255*, 10687–10694.

Meites, L., & Meites, T. (1965) *Anal. Chem. 20*, 984.

Patthy, L., & Smith, E. L. (1975) *J. Biol. Chem. 251*, 557–564.

Perutz, M. (1970) *Nature (London) 228*, 726–739.

Powers, D. A., Hobish, M. K., & Greaney, G. S. (1981) *Methods Enzymol. 76*, 559–577.

Ray, J., & Englander, S. W. (1986) *Biochemistry 25*, 3000–3007.

Rosa, J., & Richards, F. M. (1979) *J. Mol. Biol. 133*, 399–416.

Rosa, J., & Richards, F. M. (1981) *J. Mol. Biol. 145*, 835–851.

Willumsen, L. (1971) *C. R. Trav. Lab. Carlsberg 38*, 223–295.

Woodward, C. K., Simon, I., & Tuchsen, E. (1982) *Mol. Cell. Biochem. 48*, 135–160.