Water Catalysis of Peptide Hydrogen Isotope Exchange†

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ABSTRACT: The temperature dependence of the hydrogen–tritium and deuterium–hydrogen exchange reactions in poly(DL-alanine) has been reexamined. The results indicate a significant contribution to the observed exchange rates from the water-catalyzed reaction at pD values near pD_{min}. The activation enthalpy for water-catalyzed deuterium–hydrogen exchange in poly(DL-alanine) is found to be 21 kcal mol^{-1}. As a result, the contribution to the observed exchange rate from the water-catalyzed reaction increases with increasing temperature which in turn leads to broad, shallow pD minima and the appearance of apparent reaction orders with respect to [D^+] and [OD^-] that are substantially less than first order over an extended range of pD values. The importance of water catalysis in protein hydrogen exchange is demonstrated by a reanalysis of data for the exchange of single protons in bovine pancreatic trypsin inhibitor [Hilton, B. D., & Woodward, C. K. (1979) *Biochemistry 18*, 5834; Richarz, R., Sehr, P., Wagner, G., & Wüthrich, K. (1979) *J. Mol. Biol. 130*, 19]. The pD dependence of these protons can be explained in terms of an increased contribution from water catalysis.

Hydrogen isotope exchange is a powerful technique for studying the conformational dynamics of a protein. The majority of amide hydrogens exchange from the native protein conformation at rates that are orders of magnitude slower than those observed for the corresponding random-coil polypeptide (Barksdale & Rosenberg, 1982). The decrease in exchange rate may be attributed to the reduced accessibility of the exchange catalyst to the buried peptide sites of the protein. In addition to conformational effects, the exchange reaction is also sensitive to the chemical environment of the peptide site. So that information relevant to structural dynamics from hydrogen exchange studies can be obtained, the behavior of the chemical exchange reaction itself must be described in detail over a wide range of experimental conditions.

Studies of the exchange reaction in small model amides, polymer amides, and random-coil polypeptides [for a review, see Barksdale & Rosenberg (1982)] have established the relationship

$$k_{obsd} = k_0 + k_L[L^+] + k_{OL}K_w/[L^+] \quad (1)$$

where L denotes the appropriate hydrogen isotope, k_L and k_{OL} are rate constants for specific acid and base catalysis, respectively, K_w is the ionization constant of water, and k_0 is the rate constant for direct exchange with water.

Random-coil polypeptides such as poly(DL-alanine) and poly(DL-lysine) have been commonly employed as models of the chemical exchange reaction in proteins and have been used to estimate the effects of neighboring groups, discrete charges, temperature, and ionic strength (Barksdale & Rosenberg, 1982). Most of these studies have been confined to low temperatures and to pH values that are well removed from pH_{min}, the pH where k_{obsd} takes it minimum value. Under such conditions, the contribution to the observed rate from direct exchange with water is small, and k_0 is often neglected in eq 1:

$$k_{obsd} \simeq k_L[L^+] + k_{OL}K_w/[L^+] \quad (2)$$

However, recent work with bovine pancreatic trypsin inhibitor (BPTI) at high temperatures (Hilton & Woodward, 1978, 1979; Richarz et al., 1979) necessitates a closer examination of the water-catalyzed reaction. A comparison of the commonly used activation energies of 15 and 17 kcal mol^{-1} for the acid- and base-catalyzed exchange reactions, respectively (Englander & Poulsen, 1969; Barksdale & Rosenberg, 1982), with the only reported activation energy for the water-catalyzed reaction of 28 kcal mol^{-1} (Englander et al., 1979) suggests an increased role for water catalysis at elevated temperatures. If, in addition, we consider the possibilities for differential accessibility to the protein interior for the three catalyst species, the necessity for better knowledge about the water-catalyzed reaction becomes important.

In this paper, therefore, the pH and temperature dependences of hydrogen–tritium and deuterium–hydrogen exchange rates for poly(DL-alanine) are reexamined. On the basis of the resulting activation parameters, chemical exchange rates that include contributions from direct exchange with water have been calculated over a wide range of temperatures and pD. The significance of the results for protein hydrogen exchange studies is demonstrated by a reanalysis of the pD dependence of exchange rates for some single protons in BPTI.

Materials and Methods

Materials. Poly(DL-alanine) [PDLA type I, M_r (1–5) × 10^3] was supplied by Sigma Chemical Co. [^{14}C]Formaldehyde (51 mCi mmol^{-1}) and tritiated water (100 mCi mL^{-1}) were obtained from New England Nuclear. Polyacrylamide (P2 medium grade) was obtained from Bio-Rad Laboratories. D_2O (99.8 atom % D) was obtained from Aldrich Chemical Co. Cellulose filters (1.2 μm) were from Metricel. All other materials were either AnalaR or reagent grade as supplied by Mallinckrodt and Fisher Scientific. The scintillation cocktail was as previously described (Carter et al., 1978).

Hydrogen–Tritium Exchange. Poly(DL-alanine) (PDLA) was labeled with H^{14}CHO by reductive methylation in 0.2 M borate, pH 11.25 (Rice & Means, 1971). The reaction was allowed to proceed for 15 min at 0 °C. Tritium in-exchange was achieved by incubating [^{14}C]PDLA (^{14}C specific activity 5 μCi g^{-1}) with tritiated water buffered to pH 4.0 at 40 °C for 15 min. The polymer concentration and tritium activity of the in-exchange mixture were 20 mg mL^{-1} and 5 mCi mL^{-1}, respectively. Exchange rates were determined by using the two-column separation technique of Englander (1963) with some modification. An aliquot of the in-exchange containing approximately 2 mg of PDLA was separated from excess tritium by passage through a P2 polyacrylamide column (1.5 × 6 cm) equilibrated with 0.02 M citrate–0.05 M NaCl at the desired experimental pH at 2 °C. The eluant (out-ex-

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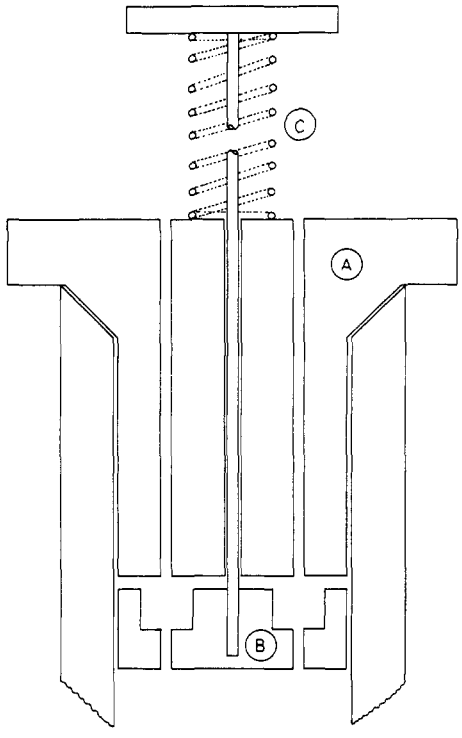

FIGURE 1: Cross section of the mixing device employed in the deuterium–hydrogen exchange studies. Details are given in the text.

change) was incubated at the desired experimental temperature, ±0.02 °C. Subsequent separations were performed at pH 3.0 and 2 °C. ^{14}C and ^{3}H activities were determined by exhaustive counting on a Beckman LS-230 liquid scintillation counter. PDLA concentrations were determined from the ^{14}C activity of polypeptide standards, the concentrations of which had been established previously by optical density measurements at 220 nm assuming an extinction coefficient of 368 M^{-1} (peptide N) (Englander & Poulsen, 1969).

Deuterium–Hydrogen Exchange. PDLA stock solutions (18 mg mL^{-1}) were prepared in 0.01 M citrate at the appropirate experimental pH and were filtered through 1.2-μm cellulose filters before use. D_2O solutions were buffered with 0.01 M citrate/DCl or KCl/DCl. The apparent D^+ activity was measured by a glass electrode calibrated with ordinary aqueous buffers. The observed pH meter readings, pH*, were then corrected to give the true D^+ activity (Glasoe & Long, 1960):

$$pH^* = pD - 0.4$$

The deuterium–hydrogen exchange reaction was monitored with the UV method (Takahashi et al., 1978; Englander et al., 1979). The replacement of hydrogen by deuterium at the peptide nitrogen causes a blue shift of the peptide absorbance envelope which produces a 5% change in absorbance at 220 nm. The accurate measurement of such small absorbance changes can be difficult. In particular, absorption of water vapor by D_2O in the cuvettes and small perturbations in temperature during mixing were found to cause a significant drift in absorbance with time. These problems were simply but effectively eliminated with the mixing device shown in Figure 1. The Teflon cap A is machined to form a seal against the inside of the quartz cuvette. Three holes are drilled through the cap. The center hole accommodates a stainless-steel rod which is attached to the Teflon mixing piston B. The piston has two sample wells drilled into it that align with the holes in the cap, through which the sample may be dispensed. A small hole is drilled through the piston at the bottom of each sample well to allow the solution to pass from one side of the piston to the other as the piston moves through the solution during mixing. The piston moves freely up and down the cuvette and was held snugly against the bottom of the cap before and after mixing by the retention spring C. D_2O solution (750 μL) was added to the cuvette, and the mixing device was put in place. Ten microliters of PDLA stock solution was carefully dispensed into the sample wells, and the whole assembly was allowed to equilibrate to the required experimental temperature in the thermostated cuvette holder of the spectrophotometer. The reaction was started by depressing the mixing piston several times. The change in absorbance at 220 nm was measured with a Cary 118 spectrophotometer interfaced to a DEC PDP11/10 computer via a Wrenchman serial line controller (Wrenchman Inc., Minneapolis, MN). The controller employs a high-level, user-oriented command language to set all Cary 118 functions and to acquire time, wavelength, and absorbance data. A FORTRAN program executing on a PDP11 computer determined the operating sequence of the spectrophotometer by sending appropriate command strings to the controller. Absorbance data were acquired by the controller at 0.3-s intervals and were averaged to give a total of 100 absorbance values over a predetermined period of time. After this period, the computer continued to take and average data at 5-min intervals. Data acquisition was terminated when two such absorbance values differed by less than 4×10^{-4} absorbance units. The last value so obtained provided the estimate of $A(\infty)$, the absorbance of the fully deuterated polypeptide.

Values of $H(t)$, the number of hydrogens remaining unexchanged at time t, were calculated according to

$$H(t) = \frac{A(t) - A(\infty)}{A(\infty)(\epsilon_H/\epsilon_D - 1)} \quad (3)$$

where $A(t)$ is the absorbance at time t, ϵ_D is the extinction coefficient of the deuterated polypeptide, and ϵ_H is the extinction coefficient of the hydrated polypeptide corrected for the instantaneous solvent perturbation that occurs on transfer to D_2O. The value of ϵ_H/ϵ_D for PDLA is 1.0485 (Englander et al., 1979). Rates were estimated by a weighted least-squares analysis of ln $H(t)$ as a function of t.

An example of the quality of hydrogen–deuterium exchange data obtained with the serial line controller is shown in Figure 2. The exchange rates were determined from the initial linear portion of the plot.

Results

Specific Acid and Base Catalysis. Values of the rate constants for specific acid- and base-catalyzed exchange can be obtained directly at pH values that are sufficiently far removed from pH_{min}, the pH at which the observed rate takes its minimum value. The values of pH_{min} and pD_{min} for PDLA are 2.9 and 3.3, respectively (Englander & Poulsen, 1969; Englander et al., 1979). In practice, values of k_H and k_{OH} for hydrogen–tritium exchange were obtained at pH values less than 2.0 and greater than 3.7, respectively. Values of k_D and k_{OD} for deuterium–hydrogen exchange were obtained at pD values of 1.5 and 5.2, respectively. The temperature dependence of k_H, k_D, k_{OH}, and k_{OD} is shown in Figure 3. Estimates of the activation parameters for the acid- and base-catalyzed reactions are given in Table I. The values of the rate constants are in good agreement with previous estimates (Englander & Poulsen, 1969; Englander et al., 1979). Values of the activation enthalpy for the base-catalyzed reaction are also in good agreement with earlier estimates by Englander's group after the enthalpy for the ionization of water is taken into account. The activation enthalpy for acid-catalyzed hydrogen-tritium

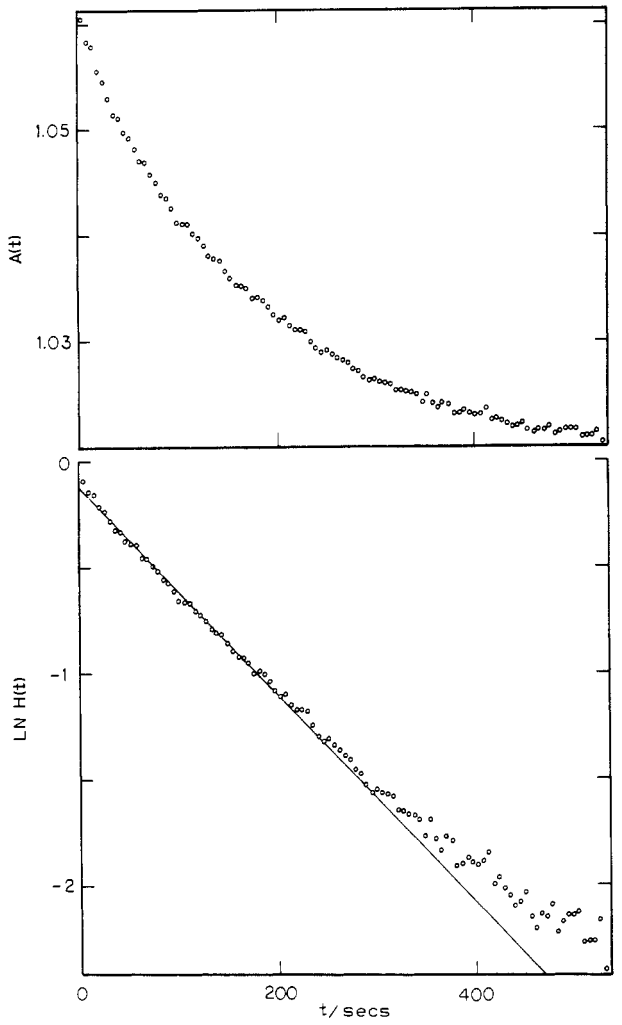

FIGURE 2: Example of the hydrogen–deuterium exchange data obtained with the Wrenchman serial line controller. Ten microliters of PDLA in H_2O was diluted into 750 μL of D_2O at pD 1.54 and 5 °C. Values of $A(t)$, the absorbance at 220 nm, were acquired at 0.3-s intervals and averaged to provide 100 data points. Values of $H(t)$ were calculated according to eq 3.

Table I: Activation Parameters for Hydrogen-Tritium (H/T) and Deuterium-Hydrogen (D/H) Exchange of Poly(DL-alanine) at 298.15 K[a]

isotope	k_L (L mol^{-1} s^{-1})	$\Delta H^{\ddagger}_L$ (kcal mol^{-1})	$\Delta S^{\ddagger}_L$ (Gibbs)	$\Delta G^{\ddagger}_L$ (kcal mol^{-1})
D/H	1.1	16.0 ± 0.2	−4.6 ± 1.6	17.4
H/T	0.5	14.4	−11.6	17.9
isotope	k_{OL} (L mol^{-1} s^{-1})	$\Delta H^{\ddagger}_{OL}$ (kcal mol^{-1})	$\Delta S^{\ddagger}_{OL}$ (Gibbs)	$\Delta G^{\ddagger}_{OL}$ (kcal mol^{-1})
D/H	1.9×10^8	1.2 ± 0.4	−16.6 ± 2.6	6.2
H/T	7.7×10^7	4.0	−9.1	6.7
isotope	k_0 (s^{-1})	$\Delta H^{\ddagger}_0$ (kcal mol^{-1})	$\Delta S^{\ddagger}_0$ (Gibbs)	$\Delta G^{\ddagger}_0$ (kcal mol^{-1})
D/H	6.4×10^{-4}	21.0 ± 1.6	−2.6 ± 14.0	21.8
H/T	4.4×10^{-4}	15.7	−21.4	22.0

[a] The subscripts L, OL, and 0 refer to the acid-, base-, and water-catalyzed reactions, respectively. The estimates of the errors represent a 95% confidence interval.

exchange of 14 ± 4 kcal mol^{-1} is identical with that obtained previously by Englander & Poulsen (1969). However, there is a large discrepancy in the activation enthalpy for acid-catalyzed deuterium–hydrogen exchange, reported here as 16.0 kcal mol^{-1}, compared with the value of 12 kcal mol^{-1} reported by Englander et al. (1979).

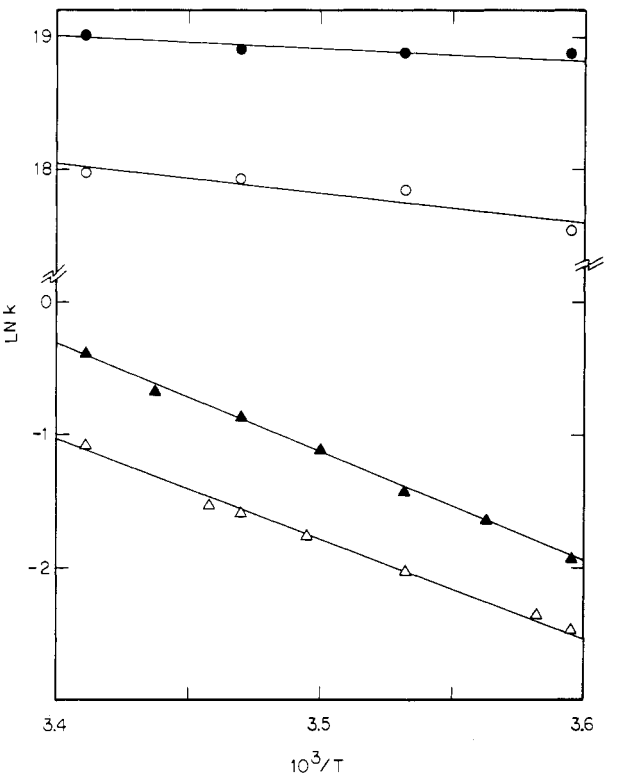

FIGURE 3: Temperature dependence of the acid- and base-catalyzed exchange rate in PDLA. (▲) k_D; (△) k_H; (●) k_{OD}; (○) k_{OH}. k is given in liters per mole per second.

Direct Exchange with Water. Estimates of k_0 for hydrogen–tritium and deuterium–hydrogen exchange over a wide range of temperatures were obtained from measurements at pH 3.0 and pD 3.3, respectively. The contributions from specific acid and base catalysis were calculated at each temperature from the activation parameters given in Table I together with the values of K_w for H_2O and D_2O given by Covington et al. (1966). Estimates of k_0 were obtained by substitution and rearrangement of eq 1. The temperature dependence of k_0 is shown in Figure 4, and the activation parameters are listed in Table I. The values of k_0 for hydrogen–tritium and deuterium–hydrogen exchange at 20 °C are 2.5×10^{-4} and 3.1×10^{-4} s^{-1}, respectively, in reasonably good agreement with the value of 4.4×10^{-4} s^{-1} obtained by Englander et al. (1979) for deuterium–hydrogen exchange. However, the value of the activation enthalpy, $\Delta H^{\ddagger}_0$, for the deuterium–hydrogen exchange reaction, determined here as 21 kcal mol^{-1}, is rather less than the value of 28 kcal mol^{-1} obtained by Englander et al. (1979). The estimate of $\Delta H^{\ddagger}_0$ by Englander et al. (1979) was derived from data at only two temperatures and in addition involved the exchange of different isotope pairs at the two temperatures, a fact that neglects possible solvent isotope and kinetic isotope effects.

Finally, note that the value of $\Delta H^{\ddagger}_0$ for the hydrogen–tritium exchange reaction is surprisingly low, only 15.7 kcal mol^{-1}. The hydrogen–tritium exchange technique is not very well suited to measuring the fast exchange rates necessary for accurate determinations of k_0 at high temperatures, a fact that could cause $\Delta H^{\ddagger}_0$ to be underestimated.

Discussion

The results given in Table I indicate that the water-catalyzed reaction makes a significant contribution to the observed ex-

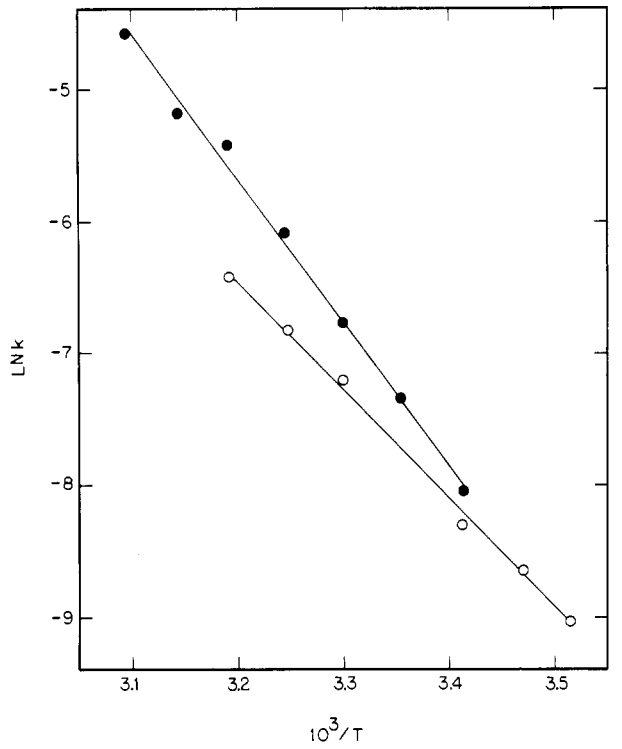

FIGURE 4: Temperature dependence of the water-catalyzed exchange rate in PDLA. (●) Deuterium–hydrogen exchange rates; (○) hydrogen–tritium exchange rates. k is given in reciprocal seconds.

change rate. The relative contributions of the acid-, base-, and water-catalyzed hydrogen–tritium exchange reaction calculated at 25 °C and pH_{min} 2.91 are 37%, 37%, and 26%, respectively. The corresponding values for the deuterium–hydrogen exchange reactions at 25 °C and pD_{min} 3.32 are 31%, 31%, and 38%. In addition, the large value of $\Delta H^{\ddagger}_0$ indicates that at higher temperatures k_0 actually dominates the observed exchange rate at pD values near pD_{min}. As a result, the pD minimum becomes broad and shallow as the temperature is raised, an effect that has been observed in the deuterium–hydrogen exchange of single protons in bovine pancreatic trypsin inhibitor by NMR (Hilton & Woodward, 1978, 1979). The relative contributions of acid, base, and water catalysis to the observed exchange rate for PDLA, calculated from the activation parameters given in Table I, are shown in Figure 5. The increased contribution from water catalysis about pD_{min} as the temperature is raised is readily apparent. A second consequence of the increased contribution from water catalysis is the appearance of apparent reaction orders with respect to $[D^+]$ and $[OD^-]$ that are substantially less than first order over an extended range of pD values about pD_{min}. The value of the apparent order of the reaction with respect to $[D^+]$ is given by

$$\frac{-\text{d log } k_{obsd}}{\text{dpD}} = \frac{k_D[D^+] - k_{OD}K_w/[D^+]}{k_0 + k_D[D^+] + k_{OD}K_w/[D^+]} \quad (4)$$

The plot of $-\text{d log } k_{obsd}/\text{dpD}$ as a function of pD at different temperatures is shown in Figure 6. It is apparent that at 0 °C a shift in pD of ±1.5 units from pD_{min} is sufficient to ensure first-order behavior with respect to $[D^+]$ or $[OD^-]$, while at 80 °C it is necessary to shift the pD at least ±2.5 units from pD_{min} before the kinetics become first order with respect to $[D^+]$ or $[OD^-]$.

Finally, it should be noted that the observed activation enthalpy for the exchange reaction will also vary with pD and temperature as the relative contributions from acid, base, and

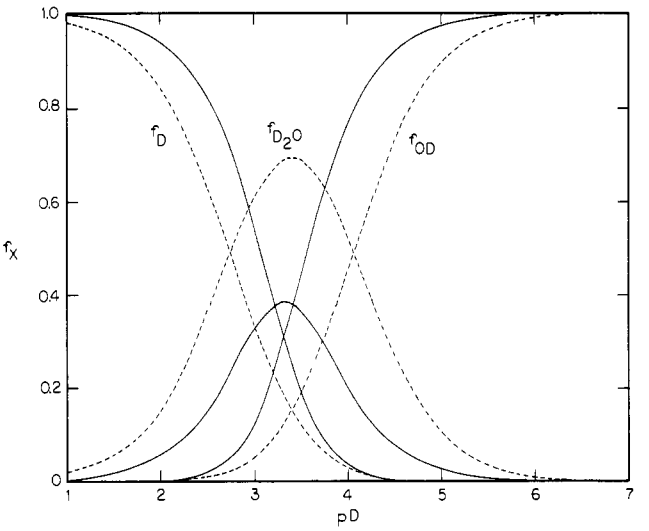

FIGURE 5: Relative contributions of acid (f_D), base (f_{OD}), and water (f_{D_2O}) catalysis to the observed exchange rate in PDLA at 25 (—) and 70 °C (---). Values of f_D, f_{OD}, and f_{D_2O} were calculated from the activation parameters given in Table I: $f_D = k_D[D^+]/k_{obsd}$, $f_{OD} = k_{OD}K_w[D^+]k_{obsd}$, and $f_{D_2O} = k_0/k_{obsd}$.

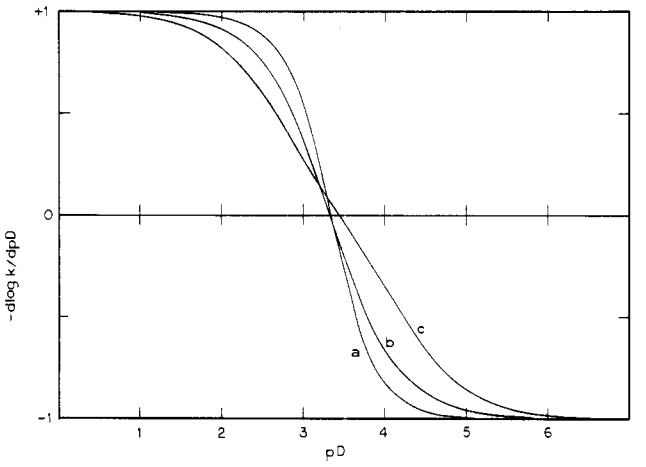

FIGURE 6: Apparent reaction order, $-\text{d log } k/\text{dpD}$, with respect to $[D^+]$ as a function of pD at (a) 0, (b) 40, and (c) 80 °C calculated according to eq 4.

water catalysis change. The observed activation enthalpy is given by

$$\Delta H^{\ddagger}_{obsd} = \frac{\Delta H^{\ddagger}_0 k_0 + \Delta H^{\ddagger}_D k_D[D^+] + (\Delta H_w + \Delta H^{\ddagger}_{OD})k_{OD}K_w/[D^+]}{k_0 + k_D[D^+] + k_{OD}K_w/[D^+]} \quad (5)$$

The behavior of $\Delta H^{\ddagger}_{obsd}$ as a function of pD and temperature is shown in Figure 7. As the temperature is raised, the increased contribution from water catalysis about pD_{min} is revealed by a pronounced maximum in $\Delta H^{\ddagger}_{obsd}$. Note also that at high pD values, the limiting value of $\Delta H^{\ddagger}_{obsd}$ decreases as the temperature is raised due to the large negative ΔC_p associated with the ionization of water.

It is apparent that an Arrhenius plot of the observed exchange rate determined at a pD value near the pD_{min} will be curved although on the basis of the activation parameters given here such curvature will only be slight.

Water Catalysis in Protein Hydrogen Exchange. The identification of a large contribution to peptide hydrogen exchange from the water-catalyzed reaction described here is of particular significance to an understanding of hydrogen exchange mechanisms in proteins. The pH dependence of

Table II: Rate Constants for the Exchange of Seven Protons in BPTI at 68 °C[a]

	Tyr-23	Phe-45	Phe-22	Phe-33	Tyr-21	Gln-31	Arg-20
$k_0 \times 10^5$ (s^{-1})	2.22	4.13	2.01	2.02	1.88	2.70	2.54
$k_D \times 10^3$ (L mol^{-1} s^{-1})	4.89	5.87	3.68	2.10	4.39	3.37	4.53
$k_{OD} \times 10^{-2}$ (L mol^{-1} s^{-1})	5.11	6.16	4.80	4.83	6.10	6.57	13.3
pD_{min}	4.32	4.32	4.27	4.15	4.26	4.18	4.09
$k_{min} \times 10^5$ (s^{-1})	2.27	4.19	2.05	2.05	1.93	2.75	2.61
k_0/k_{min}	0.98	0.99	0.98	0.99	0.97	0.98	0.97

[a] Constants obtained from a fit of the data of Hilton & Woodward (1979) to eq 1. A value of $K_w = 2.2 \times 10^{-14}$ at 68 °C was assumed (Covington et al., 1966).

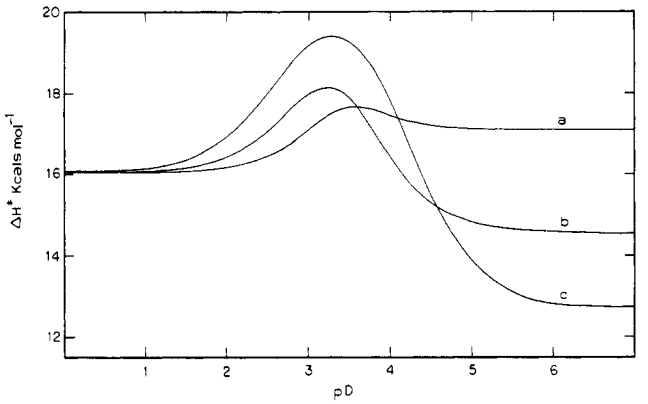

FIGURE 7: Apparent activation enthalpy as a function of pD at (a) 0, (b) 40, and (c) 80 °C calculated according to eq 5.

exchange rates in proteins is often observed to deviate from a simple first-order dependence on $[H^+]$ and $[OH^-]$ (Willumsen, 1971; Woodward & Hilton, 1979; Hilton & Woodward, 1979) and displays pH_{min} values that vary considerably from the value that characterizes the exchange of PDLA (McBride-Warren & Mueller, 1972; McBride-Warren & Richert, 1976; Hilton & Woodward, 1979). The latter can be explained on the basis of inductive effects of neighboring peptide groups (Molday et al., 1972), by the polarity of the exchange environment (Leichtling & Klotz, 1966), and by electrostatic effects (Kim & Balwin, 1982). However, the reasons for less than first-order pH dependencies observed for exchange from proteins in the folded conformation are still unclear. Indeed, even the random-coil heteropolypeptide oxidized ribonuclease displays a pH dependence that is less than first order (Molday et al., 1972).

It should be noted that in many cases these deviations appear only in the pH range 2–6, close to the pH_{min} for exchange. This is exactly the pH region where deviations would be expected if the water-catalyzed reaction were making significant contributions to the observed exchange.

A particularly striking example is provided by the studies of Hilton & Woodward (1979) and Richarz et al. (1979) on the exchange of single protons in BPTI by NMR. The data consist of the complete pD dependence of exchange rates for the slowest exchange protons at a number of temperatures. In most cases, the pD minima are broad and shallow and display apparent reaction orders with respect to $[D^+]$ and $[OD^-]$ that are less than first order. The pD dependence is further complicated, for some residues, by an anomalous pD dependence between pD 7.0 and 9.0, and by an apparent increase in reaction order with respect to $[OD^-]$ as the temperature is decreased.

There is no general agreement as to the mechanisms of exchange in BPTI. Hilton & Woodward (1979) explained the pD and temperature dependence of exchange in terms of a two-process model with different pD dependences and activation energies for each process. The pD dependence near the pD_{min} at 68 °C was attributed to two opposing effects. First, the acid-catalyzed exchange rate is accelerated relative to that of the base as protein unfolding is increased with decreasing pD. Second, there is a decrease in the rate of the chemical exchange step, for which a pH^*_{min} ($pH^* = pD - 0.4$) of 2.8 is assumed. The net result of these effects is to increase the value of the apparent pH^*_{min} and give a nearly pD-independent region between pH^* 3 and 4 (Hilton et al., 1981). The anomalous pD dependence between pD 7 and 9 is accounted for in terms of a transition between two processes with different pD dependences and activation energies. Wagner & Wüthrich (1979) have explained the pD dependence of exchange rates in terms of a multistate model in which the populations of accessible states of different degrees of ionization vary with pD in a manner consistent with the pK values of groups on the protein. Both models assume that direct exchange with water is negligible. However, the results described for PDLA suggest that this assumption may be incorrect, particularly at high temperatures where certain qualitative features of the pD dependences of exchange rates in BPTI are very similar to the behavior observed in the present work for PDLA, in which a large contribution to the observed exchange rate from the water-catalyzed reaction is evident. Hilton & Woodward (1979) have determined the complete pD dependence of exchange rates for seven NH protons in BPTI at 68 °C. The data were fitted to the following equation:

$$k_{obsd} = k_1[D^+]^{0.81} + k_2[D^+]^{-0.51} + k_3 \qquad (6)$$

We have reanalyzed these data with eq 1, which in contrast to eq 6 predicts a simple first-order dependence on $[D^+]$ and $[OD^-]$. A weighted linear least-squares analysis provided estimates of k_0, k_D, and k_{OD}, given in Table II, for each resonance. The weights were set equal to k_{obsd}^{-2}, consistent with assuming an approximately equal error in ln k. The quality of the fits of the data to eq 1, shown in Figure 8, is as good as that obtained by Hilton & Woodward (1979) with eq 6 and suggests that the pD dependence of the exchange rates at 68 °C could be quite "normal" with first-order dependences on $[D^+]$ and $[OD^-]$ and a major contribution to the exchange from water catalysis. Hilton & Woodward (1979) have also determined the exchange rates for the same protons at 51 and 33 °C at pD >7. The limited pD range makes it difficult to estimate values of k_0 reliably. Nevertheless, examination of Figure 1 of Hilton & Woodward (1979) reveals a clear increase in the slope, d log k_{obsd}/dpD, with decreasing temperature for Arg-20, Glu-31, Phe-33, and Phe-45, consistent with an increase in the contribution from water catalysis as the temperature is raised. The pD dependence of exchange rates for Tyr-21, Phe-22, and Tyr-23 at 51 °C is anomalous between pD 7 and 9.5 and has been accounted for in terms of a transition between two exchange processes with different pD and temperature dependences (Hilton & Woodward, 1979). Unfortunately, the pD range is not sufficient to accurately determine the position of the deviation on the pD scale.

Table III[a]

	Tyr-23	Phe-45	Phe-22	Tyr-21	Phe-33	Arg-20	Gln-31	Gln-31	Ile-18	Ile-18	Gly-36	Tyr-35	Tyr-35
temp (°C)	45	45	45	45	45	45	45	22	45	22	45	45	22
$k_0 \times 10^8$ (s^{-1})	4.18	8.40	2.39	3.19	3.30	2.51	4.99	0.68	4.50	0.75	583	14.7	0.59
$k_D \times 10^7$ (L mol^{-1} s^{-1})	9.29	20.0	12.6	8.25	2.62	36.1	71.3	11.2	237.0	21.7	1010	210.5	7.1
k_{OD} (L mol^{-1} s^{-1})	1.56	107.1	0.21	1.45	77.6	43.9	55.4	18.5	1200	170	1.03×10^6	6450	410
pD_{min}	4.01	3.26	4.51	4.00	2.88	3.58	3.68	3.89	3.27	3.56	2.12	2.88	3.12
$k_{min} \times 10^8$ (s^{-1})	4.20	8.62	2.40	3.21	3.37	2.70	5.29	0.71	7.06	0.87	737	20.3	0.70
k_o/k_{min}	>0.99	0.97	>0.99	>0.99	0.98	0.93	0.94	0.96	0.64	0.86	0.79	0.73	0.85

[a] Rate constants for the exchange of 10 protons in BPTI at 22 and 45 °C obtained from a fit of the data of Richarz et al. (1979) to eq 1. Values for K_w at 22 and 45 °C of 1.05×10^{-15} and 5.74×10^{-15} were assumed, respectively (Covington et al., 1966).

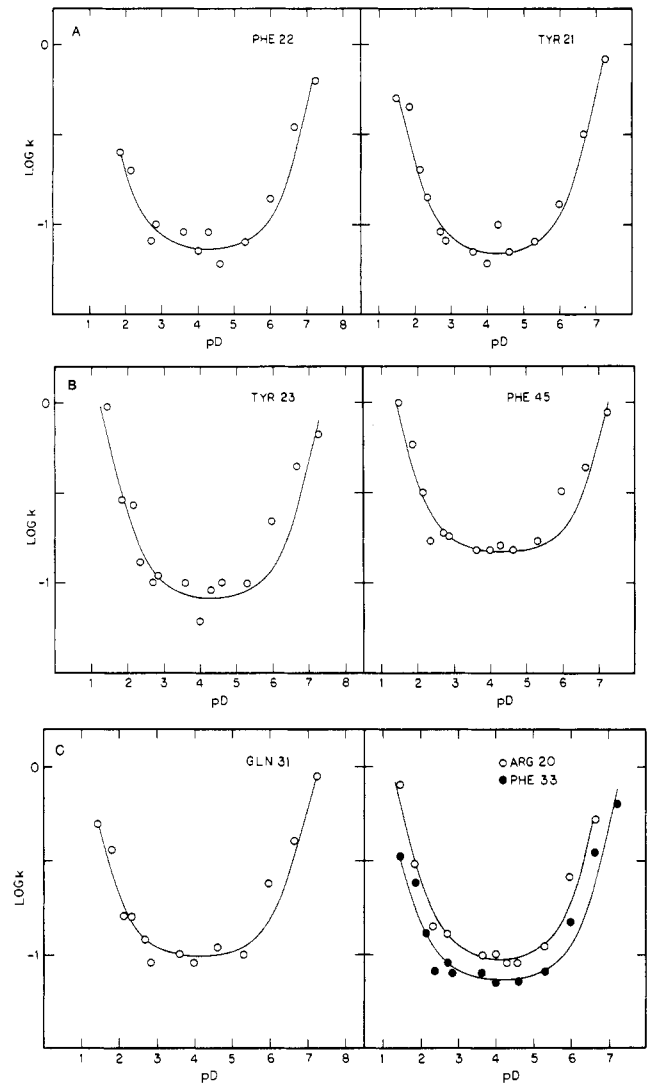

FIGURE 8: pD dependence of the exchange rates of seven protons in BPTI at 68 °C. The data points are from Hilton & Woodward (1979). The fitted curves are calculated according to eq 1 with the rate constants given in Table II. k is given in reciprocal hours.

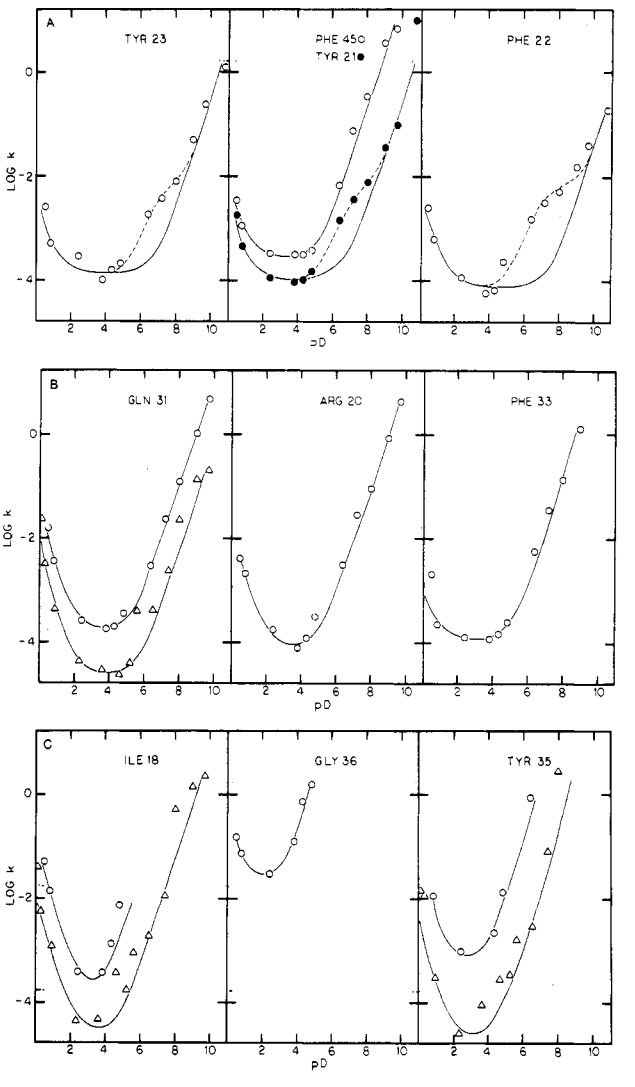

FIGURE 9: pD dependence of the exchange rates of 10 protons in BPTI at (△) 22 and (○) 45 °C. The data points are from Richarz et al. (1979). The solid curves are calculated according to eq 1 with the rate constants given in Table III. The dashed curves are calculated according to eq 9 with the rate constants given in Table IV together with $pK_A = 7.0$. k is given in reciprocal hours.

Single proton exchange data for BPTI have also been obtained at 22 and 45 °C by Richarz et al. (1979). As before, the pD dependences deviate from the behavior predicted by eq 2 and were reanalyzed with eq 1. Values of k_0, k_D, and k_{OD} are listed in Table III. The fits to the data are shown in Figure 9. The inclusion of a term to account for the water-catalyzed reaction in eq 1 appears to describe the pD dependence of most of the protons over a broad pD range. However, deviations from this simple behavior are again apparent in the pD dependences for Tyr-21, Phe-22, and Tyr-23 at 45 °C. Hilton & Woodward (1979) suggest that these deviations reflect the transition between two processes of exchange, which is proposed to account for the pD and temperature dependence of exchange rates. However, the observed deviations are also similar to the pD–rate profiles expected from discrete charge effects (Barksdale & Rosenberg, 1982; Wagner & Wüthrich, 1979). The ionization of a group in the

Table IV: Rate Constants Obtained from a Fit of the Data of Richarz et al. (1979) to Equation 9[a]

	Tyr-21	Phe-22	Tyr-23
k_{OD}^{A} (L mol^{-1} s^{-1})	1.13	0.18	1.28
$k_{OD}^{AD^+}$ (L mol^{-1} s^{-1})	38.3	33.1	37.2
$k_D \times 10^7$ (L mol^{-1} s^{-1})	8.25	12.6	9.29
$k_0 \times 10^8$ (s^{-1})	3.19	2.39	4.18
pD_{min}			
$k_{min}^{A} \times 10^8$ (s^{-1})	3.21	2.40	4.20
k_0/k_{min}^{A}	>0.99	>0.99	>0.99

[a] A value of $K_w = 5.74 \times 10^{-15}$ at 45 °C was assumed (Covington et al., 1966). In each case, $pK_A = 7.0$. k_{min}^{A} refers to the minimum rate calculated for species A.

protein with proton dissociation constant K_A gives rise to two species:

$$AD^+ \rightleftharpoons^{K_A} A + D^+$$

with different hydrogen exchange rates for particular sites within the protein either directly, by affecting the chemical exchange rate, or indirectly, by affecting the relative accessibility of the sites to the catalyst species.

The observed exchange rate is given by

$$k_{obsd} = \alpha k_{obsd}^{A} + (1 - \alpha) k_{obsd}^{AD^+} \quad (7)$$

where α denotes the extent of proton dissociation, $\alpha = K_A/(K_A + D^+)$, and k_{obsd}^{A} and $k_{obsd}^{AD^+}$ are given by

$$k_{obsd}^{A} = k_0^{A} + k_D^{A}[D^+] + k_{OD}^{A} K_w/[D^+] \quad (8a)$$

and

$$k_{obsd}^{AD^+} = k_0^{AD^+} + k_D^{AD^+}[D^+] + k_{OD}^{AD^+} K_w/[D^+] \quad (8b)$$

Analysis of the data for Tyr-21, Phe-22, and Tyr-23 employed a simplified form of eq 7 and 8 in which k_0 and k_D for both protein species were constrained to the values obtained with eq 1:

$$k_{obsd} = k_0 + k_D[D^+] + [\alpha k_{OD}^{A} + (1 - \alpha) k_{OD}^{AD^+}] K_w/[D^+] \quad (9)$$

Distinct values of $k_D^{AD^+}$ and k_D^{A} cannot be obtained, since the contributions to the observed rate from the acid-catalyzed reaction are negligible at pD values above 6.0. When k_0^{A} and $k_0^{AD^+}$ were included in the analysis, the estimates of k_0^{A} and $k_{OD}^{AD^+}$ were found to be highly covariant as pK_A was allowed to vary, with k_0^{A} often adopting negative values. Analysis of the data with eq 9 provides the values of k_{OD}^{A} and $k_{OD}^{AD^+}$ which are given in Table IV together with an estimate of $pK_A \simeq 7.0$. The fits to the data are shown in Figure 9. It is apparent that eq 9 can account very well for the observed pD dependence.

The identity of the ionizable group assumed in the previous analysis deserves further comment. The pK_A values of all groups that titrate at pD values below 12.0 have been determined for BPTI. They include five carboxyl groups, the amino terminus, and eight lysine and tyrosine groups (Wagner & Wüthrich, 1979). The ionization of the amino terminus with a pK_A of 7.8–8.2 at 25 °C (Brown et al., 1978) is the most obvious source of the deviations observed in the hydrogen exchange rates. This assignment is supported by the following evidence. First, the ionization of α-amino groups is often associated with large, positive enthalpy changes, so that the pK_A of 7.0 obtained from hydrogen exchange studies at 45 °C is not inconsistent with the pK_A value of 7.8–8.2 observed for the α-amino group at 25 °C. The deviations that range from pD 6.5 to pD 9.5 at 45 °C are also observed in the 51 °C data for the same residues. However, the data do not extend to sufficiently low pD values to accurately determine the extent of the deviation. Second, the transamination of the amino terminus is known to affect the hydrogen exchange rates of the slowly exchanging amide protons in BPTI (Brown et al., 1978). Finally, as noted by Brown et al. (1978), Tyr-23 is located relatively close to the N-terminal residue, arginine-1, in the X-ray crystal structure (Deisenhofer & Steigemann, 1975) and shows pD-dependent chemical shifts that appear to be related to the ionization of the amino terminus. The alternative analysis of the BPTI data given here accounts very well for the broad, shallow pD minima, the values of d log k_{obsd}/dpD observed near pD_{min}, and the variation in slope on the basic side of the pD–rate profile with temperature. In addition, eq 1 provides meaningful values of the rate constants for the acid-, base-, and water-catalyzed reactions by eliminating fractional reaction orders with respect to $[D^+]$ and $[OD^-]$.

Contribution of Water Catalysis to the Observed Exchange Rates. One feature of the results that deserves particular emphasis concerns the size of the contribution to the observed exchange rate from the water-catalyzed reaction. A comparison of the values of k_0 and k_{min}, given in Tables II–IV, for the slowest exchanging protons located in the central portion of the β-sheet structure (Arg-20, Tyr-21, Phe-22, Tyr-23, Gln-31, Phe-33, and Phe-45) reveals that the water-catalyzed reaction accounts for more than 93% of the observed exchange at pD_{min} at all temperatures that have been examined. The contribution to the observed exchange from water catalysis for Ile-18, Gly-36, and Tyr-35, residues that are located at one end of the β sheet, varies from 64% to 86%. As noted previously, the corresponding contribution for PDLA is 69% at 70 °C and 38% at 25 °C (Figure 5). The marked increase in the contribution to exchange by water catalysis in BPTI compared to PDLA is not readily explained in terms of the chemical exchange reaction itself, but suggests instead a differential accessibility of the BPTI exchange sites to D_2O relative to their accessibility to the charged species, D^+ and OD^-. The relative contributions to exchange by the uncharged water and charged water ions serve as a probe of the environment of the exchange sites. It is apparent, for example, that the central region of the β-sheet core is far less accessible to the charged catalyst species than the end region of the β sheet although the rates for water-catalyzed exchange of Ile-18 and Tyr-35 are similar to those observed for the central β-sheet protons. The notion that charged species are to some extent preferentially excluded from the hydrophobic core of a protein is not unreasonable.

The increased contribution to exchange from water catalysis in BPTI also results in values of d log k_{obsd}/dpD < 1 over a substantially wider range of pD values than is observed in PDLA. The pD value necessary to secure first-order behavior with respect to $[OD^-]$ is given by rearrangement of eq 4 with the assumption that $k_{OD}K_w/[D^+] \gg k_D[D^+]$:

$$[D^+] = k_{OD}K_w(1 - C)/(Ck_0) \quad (10)$$

where C is the apparent order with respect to $[OD^-]$. For example, setting $C = 0.999$ and substituting the values of k_{OD}, k_0, and K_w for, for example, Phe-45 indicate that first-order behavior with respect to $[OD^-]$ is achieved only when pD >8.1 at 45 °C or pD >9.5 at 68 °C.

Finally, the difference in the relative contributions of the water-catalyzed reaction to exchange in BPTI and PDLA indicates that exchange occurs from a conformation that does not resemble a random coil in bulk water under the conditions examined here. This result is interesting, in view of the ob-

servation that exchange of the slowest protons in lysozyme occurs from states that thermodynamically resemble the activated complex for unfolding more closely than the fully unfolded state itself (Gregory et al., 1982).

The alternative analysis of the BPTI data presented here clearly demonstrates the importance of the water-catalyzed reaction. The contribution of water catalysis to the hydrogen exchange kinetics of other proteins remains to be elucidated. However, there is little reason to doubt that its contribution to protein hydrogen exchange is quite general.

Registry No. Poly(DL-alanine), 25281-63-4; poly(DL-alanine), SRU, 26283-00-1; BPTI, 9087-70-1; Tyr, 60-18-4; Gln, 56-85-9; Arg, 74-79-3; Ile, 73-32-5; Gly, 56-40-6; Phe, 63-91-2; hydrogen, 1333-74-0; deuterium, 7782-39-0; tritium, 10028-17-8; water, 7732-18-5.

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Dimeric Hemoglobin of the Bivalve Mollusc *Anadara broughtonii*: Complete Amino Acid Sequence of the Globin Chain†

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ABSTRACT: The complete amino acid sequence of a dimeric hemoglobin (HbI) from the marine bivalve mollusc *Anadara broughtonii* was determined by sequencing of the intact chain and peptide fragments produced by cleavage at two asparaginylglycine bonds and at methionyl, arginyl, and tryptophanyl residues. The clam hemoglobin consists of two identical polypeptide chains. The globin chain has 146 amino acid residues with a proline at the NH_2 terminus and a leucine at the COOH terminus. The calculated molecular mass of the native hemoglobin was 32945 daltons. The clam hemoglobin contains only two histidine residues, which correspond to the distal and proximal heme-linked positions. Compared with human β chain, an additional segment of seven residues is present in the NH_2-terminal region and also five less residues in the COOH-terminal region. Although such an amino-terminal elongation has been known to be characteristic of hemoglobins from the most primitive living vertebrates *Cyclostomata*, a very similar structure was found to occur in the hemoglobin from the primitive invertebrate arcid clam.

Invertebrate respiratory pigments having roles as oxygen carriers include a variety of metal–proteins (e.g., hemoglobin, hemocyanin, hemerythrin, and chlorocruorin).

Clams of the primitive family Arcidae (the so-called blood clam) have intracellular hemoglobin in the hemolymph. Hemoglobins of a few species of Arcidae (*Anadara satowi*, *Anadara broughtonii*, *Anadara senilis*, and *Scapharca inaequivalvis*) consist of two components, i.e., dimeric HbI and tetrameric HbII.[1] Both components bind oxygen cooperatively, but with no alkaline Bohr effect (Ohnoki et al., 1973; Furuta et al., 1977; Djangmah et al., 1978; Chiancone et al., 1981).

We have examined the correlation between structure and function of *A. broughtonii* hemoglobins. The major findings are as follows: (1) HbII on oxy-liganded form has an $\alpha_2\beta_2$ structure which is rare among other invertebrates. On deoxygenated form HbII shows polymerization to a highly aggregated state. (2) HbI has two like chains, which are different from either of α and β chains (Furuta et al., 1977, 1980, 1981).

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[1] Abbreviations: Hb, hemoglobin; TosPheCH$_2$Cl, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine; DMAA, dimethylallylamine; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate.

0006-2960/83/0422-0917$01.50/0