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Influence of Charge on the Rate of Amide Proton Exchange†

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ABSTRACT: Nearest-neighbor inductive effects are known to influence amide proton exchange. Here we show that long-range electrostatic interactions also have a significant effect on the rate of amide proton exchange. We have measured the effects of salt (NaCl) on the exchange rates of the neutral polypeptide poly(DL-alanine) (PDLA) and of the positively charged polymer poly(DL-lysine) (PDLL). Our results show that, while exchange from PDLA is insensitive to ionic strength, exchange from PDLL is extremely sensitive to salt concentration. In the acid-catalyzed exchange of PDLL, the exchange rate increases approximately 3-fold between ionic strengths of 60 mM and 1 M. In base-catalyzed exchange, there is a decrease in exchange rate of almost 50-fold between ionic strengths of 2 mM and 1 M. As expected, salt produces a shift of the pH_{min} (the pH at which exchange is slowest) for PDLL but not for PDLA. Our results show a salt-dependent shift of 1.3 pH units in the pH_{min} of PDLL between 0 and 2 M NaCl. These results unambiguously demonstrate a major effect of charge on the kinetics of amide proton exchange in unstructured polypeptides. We have also done some simple electrostatic calculations for the well studied protein basic pancreatic trypsin inhibitor (BPTI). Our calculations show some of the anomalous features of the pH dependence curves measured for individual amide protons of BPTI [Richarz, R., Sehr, P., Wagner, G., & Wüthrich, K. (1979) *J. Mol. Biol. 130*, 19–30], although the overall agreement is poor, indicating that additional factors (probably including the local electrostatic environment) affect the pH dependence curve. An obvious conclusion of this work is that long-range electrostatic effects, which alter the pH in the vicinity of a macromolecule, must be taken into account when interpreting proton exchange data.

Amide proton exchange was introduced as a method of studying protein conformation by Hvidt & Linderstrøm-Lang (1954). The method is useful for studying the conformation and conformational fluctuations of a protein since the rate of amide proton exchange is greatly reduced in native proteins (by as much as 10^8) when the amide proton is hydrogen bonded or when it is otherwise protected from solvent. Recently, amide proton exchange has been used to (a) study conformational fluctuations in lysozyme (Knox & Rosenberg, 1980) and ribonuclease S (Rosa & Richards, 1979, 1981), (b) measure the dissociation constants of hemoglobin (Ide et al., 1976) and of ribonuclease S (Schreier & Baldwin, 1976, 1977), (c) study ligand-induced conformational changes in hemoglobin (Englander et al., 1980), and (d) probe the structures of intermediates in the folding of ribonuclease A (Schmid & Baldwin, 1979; Kim & Baldwin, 1980). The NMR[1] assignment of the slowly exchanging amide protons in BPTI by Dubs et al. (1979) has made it possible to investigate the mechanisms of amide proton exchange in a protein by analyzing the exchange rates of individual amide protons in BPTI and its derivatives (Wüthrich et al., 1980, and references cited therein).

In unstructured peptides, neighboring groups have a major effect on the kinetics of amide proton exchange. The importance of inductive effects was suggested by Leichtling & Klotz (1966), and an important advance was made when the inductive effects of the various amino acid side chains were quantitated by Molday et al. (1972). In general, electron-withdrawing neighbors, which make the amide a stronger acid, are found to enhance base-catalyzed exchange and decrease the acid-catalyzed exchange rate.

In this paper we demonstrate that long-range electrostatic interactions also have a significant effect on amide proton exchange rates. This conclusion is based on a comparison of the effects of NaCl on the exchange rates of PDLA (a neutral polypeptide) and the positively charged polypeptide PDLL. We also show, using a smeared-charge model (Linderstrøm-Lang, 1924; Tanford, 1962), that the effects of charge can be substantial in amide proton exchange from proteins. In particular, we have made calculations for BPTI and have

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[1] Abbreviations: PDLA, poly(DL-alanine); PDLL, poly(DL-lysine); BPTI, basic pancreatic trypsin inhibitor; pH_{min}, the pH at which proton exchange is slowest; NMR, nuclear magnetic resonance; pH_{local}, the pH in the vicinity of a macromolecule; pH_{meas}, the experimentally measured pH of the bulk solution; NMA, *N*-methylacetamide; C_{local}, concentration of condensed counterions surrounding a polyelectrolyte; DNA, deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

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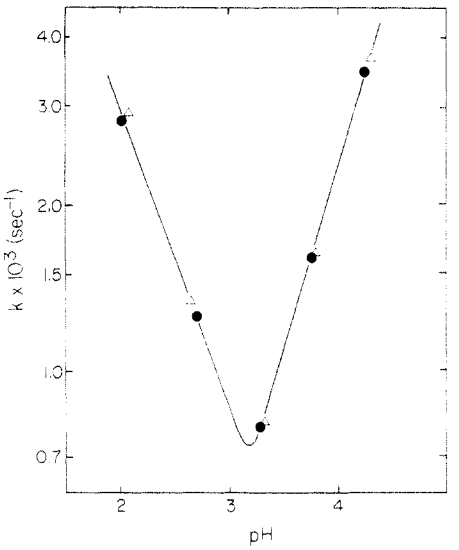

FIGURE 1: pH dependence of amide proton exchange in PDLA: (●) 0 mM NaCl and 2 mM buffer, 20.0 °C; (△) 2.0 M NaCl and 2 mM buffer, 20.0 °C.

compared the results with the data obtained by Wüthrich and co-workers (Richarz et al., 1979; Wüthrich et al., 1980). Our calculations show that some qualitative features of the pH dependence curves measured for individual amide protons in BPTI can be predicted with this approximate smeared-charge model.

Materials and Methods

PDLA was from Miles (lot A168), and PDLL hydrobromide was from Sigma (type VIB, lot 87C-5035). D_2O used, from Liquid Carbonic, was 99.7% pure. Buffers (analytical grade) were sodium oxalate, glycine, and sodium formate. All solutions contained 2 mM buffer salt equivalents (i.e., for oxalate, a dicarboxylic acid, a 1 mM concentration of the acid was used). The pH of the buffered solutions was adjusted with DCl and NaOD.

Peptide group hydrogen–deuterium exchange was monitored with the ultraviolet spectrophotometric technique developed by Englander et al. (1979). The measurements were made with a Cary 118 spectrophotometer with jacketed cell holders. Absorbance changes were recorded at 220 nm (slit = 0.2 mm) with 0.7 zero suppression and 0.05 full scale. To initiate the exchange reaction, we added an aliquot of the polymer [37 μL of PDLA (~4 mg/mL) or 5 μL of PDLL (~40 mg/mL)] in H_2O to 1.0 mL of buffer in D_2O at 20.0 °C. The rate coefficients for the first-order exchange reactions were obtained from a Guggenheim plot by using Δt values greater than $t_{1/2}$ for the reaction (Guggenheim, 1926).

An advantage of the UV technique for measuring proton exchange is that the sample can be recovered after the exchange measurements have been made. This enabled us to measure accurately the pH of our final exchange solutions. The pH values reported are the readings made with a glass electrode and a Corning Model 110 pH meter, without correction for isotope effects.

Results

PDLA. Within the experimental error of our measurements (±10%), amide proton exchange from PDLA is independent of NaCl concentration from 0 mM to 2.0 M (data not shown). This is true for both the acid-catalyzed (pH 2.04, 2 mM glycine, 20.0 °C) and the base-catalyzed (pH 4.26, 2 mM formate, 20.0 °C) exchange reactions. These results are in agreement with those of Englander & Poulsen (1969), who

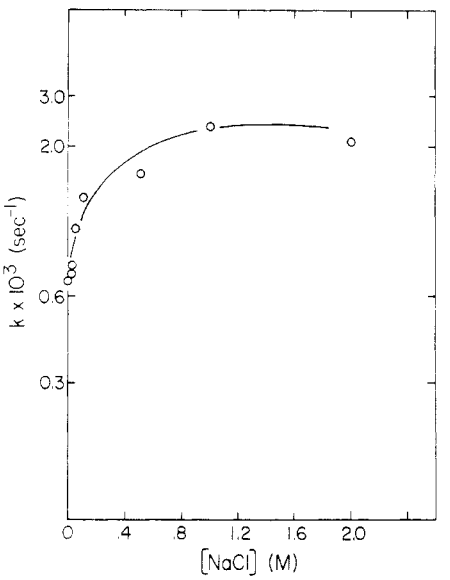

FIGURE 2: Effect of NaCl concentration on the acid-catalyzed exchange of PDLL (1 mM oxalic acid, pH 1.23 ± 0.02, 20.0 °C).

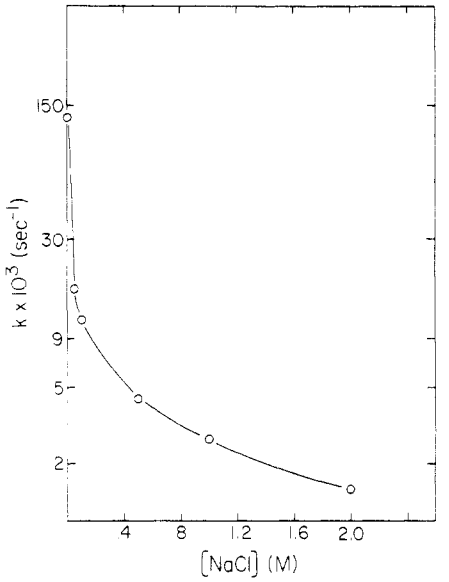

FIGURE 3: Effect of NaCl concentration on the base-catalyzed exchange of PDLL (2 mM formate, pH 3.55 ± 0.02, 20.0 °C). The rate plotted for 0 mM NaCl is extrapolated from Figure 4 because exchange is too fast to be measured directly.

also found that exchange from PDLA was insensitive to ionic strength from 50 mM to 1.0 M. Since both the acid- and base-catalyzed exchange reactions of PDLA are independent of salt concentration, the pH dependence of exchange should also be independent of salt. Figure 1 shows that the pH dependence for exchange from PDLA is the same in the presence or absence of 2 M added salt.

PDLL. In contrast to the situation with PDLA, exchange from PDLL is sensitive to salt. Figure 2 shows the salt dependence for the acid-catalyzed exchange of PDLL (pH 1.23, 1 mM oxalic acid, 20.0 °C). There is more than a 3-fold increase in the exchange rate when 1.0 M NaCl is added. Under Discussion, we give reasons why the effect of salt on the acid-catalyzed exchange reaction of PDLL is even larger than that depicted in Figure 2.

The base-catalyzed exchange of PDLL is extremely sensitive to salt concentration. The results (Figure 3) show a 100-fold decrease in the base-catalyzed exchange rate (pH 3.55, 2 mM formate, 20.0 °C) when 2.0 M NaCl is added. In the absence

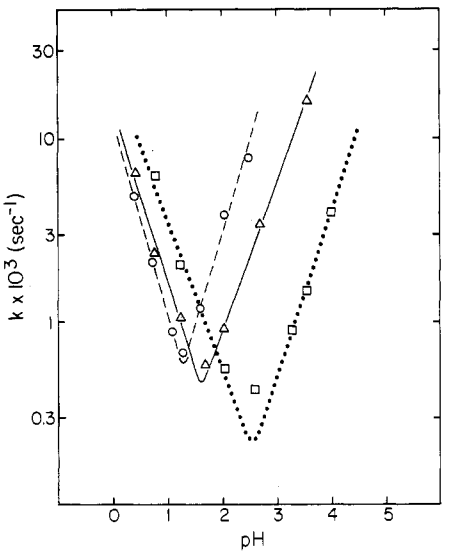

FIGURE 4: pH dependence of amide proton exchange in PDLL: (○) 0 mM NaCl and 2 mM buffer, 20.0 °C; (△) 50 mM NaCl and 2 mM buffer, 20.0 °C; (□) 2.0 M NaCl and 2 mM buffer, 20.0 °C.

of added salt, the exchange at pH 3.55, 20.0 °C, is too fast to be measured. To obtain this exchange rate, we have extrapolated the pH dependence for exchange of PDLL (at 0 mM NaCl) to pH 3.55 (see Figure 4). This extrapolated rate is plotted at 0 mM NaCl in Figure 3. All other rates were measured directly.

As NaCl is added to PDLL, the rate of the base-catalyzed reaction decreases and the rate of the acid-catalyzed reaction increases. We therefore expect a shift in the pH_{min} to higher values as salt is added to PDLL. This is what we observe (Figure 4). The pH_{min} for PDLL shifts from pH 1.25 (0 mM NaCl) to pH 1.6 (50 mM NaCl) to pH 2.6 (2.0 M NaCl). Englander & Poulsen (1969) found that the pH_{min} of PDLL at 0 °C was 2.3 when the ionic strength was 0.2 M.

BPTI. We have made some calculations to estimate the effect of electrostatic interactions on the rate of amide proton exchange in BPTI. The general considerations are as follows. (i) The pH in the vicinity of BPTI (pH_{local}) will be different from the experimentally measured pH of the bulk solution (pH_{meas}). (ii) The magnitude of the difference between pH_{local} and pH_{meas} can be estimated with a smeared-charge model (Linderstrøm-Lang, 1924; Tanford, 1962) for BPTI. This difference in pH depends on the net charge of the protein (Z) and on the ionic strength (I) of the solution, according to this model. (iii) We assume that pH_{local} determines the rate of amide proton exchange. However, the pH of the bulk solution is determined experimentally. The rate of exchange of a given amide proton, fully exposed to solvent, can be calculated at a given pH_{meas} from the value of pH_{local} and the nearest-neighbor parameters of Molday et al. (1972). In practice, few of the amide protons of a protein are fully exposed to solvent, and none of the BPTI protons studied by Richarz et al. (1979) are fully exposed. This effect is not treated here. Details of the calculations are described in the following paragraphs.

First, the net proton charge on the protein (Z) at a given pH_{meas} is determined. These calculations are made by assuming that Z depends only on the state of ionization of the titratable groups:

$$Z = \sum_i f_i z_i \qquad (1)$$

where f_i = fraction of species i that is ionized and z_i = charge of species i in the ionized form. f_i depends on the pH and on the apparent pK of the ionizable group. The apparent pKs of ionizing groups in BPTI have been determined by Wüthrich & Wagner (1979). Equation 1 gives the total net charge only so long as ions other than H^+ do not bind to the protein. Since these apparent pK values for BPTI[2] have been obtained in the absence of added salt (Wüthrich & Wagner, 1979), eq 1 should give the total net charge, to a reasonable approximation. Values of Z range from +11 at low pH to −2.5 at pH 12, with a calculated isoelectric point of pH 10.9. The experimentally measured isoelectric point of BPTI is between pH 10.5 and pH 11.0 (Chauvet et al., 1966).

The smeared-charge model for proteins has been used previously to analyze protein titration data (Tanford, 1962). The equation describing dissociation of hydrogen ions from an ionizable group, i, is

$$pH_{meas} - \log [x_i/(1 - x_i)] = (pK_{int})_i - 0.868wZ \qquad (2)$$

where x_i = fraction dissociated, pK_{int} = intrinsic pK of the ionizable group, and $0.868wZ$ takes into account the electrostatic interaction between the protein and hydrogen ion.

By assuming that the differences between the intrinsic pKs and the observed pKs are caused by differences between pH_{local} and pH_{meas}, we have

$$pH_{local} = pH_{meas} + 0.868wZ \qquad (3)$$

The electrostatic interaction parameter, w, is calculated from Linderstrøm-Lang's (1924) equation:

$$w = [\epsilon^2/(2DkTR)][1 - \kappa R/(1 + \kappa a)] \qquad (4)$$

where ϵ = electronic charge, D = dielectric constant, k = Boltzmann's constant, T = temperature, R = equivalent radius of the protein ion, κ = Debye's screening parameter, and a = distance of closest approach (usually taken as R + 2.5 Å). The radius R can be estimated from the molecular weight of the protein (Tanford, 1962):

$$(4/3)\pi R^3 = (M_r/N_0)(\bar{v}_2 + \delta_1 v_1^0) \qquad (5)$$

where M_r = molecular weight, N_0 = Avogadro's number, $\bar{v}_2$ = partial specific volume of the protein, δ_1 = grams of H_2O bound per gram of protein, and v_1^0 = partial specific volume of water. Using typical values of $\bar{v}_2$ (0.73) and δ_1 (0.2), eq 5 gives R = 13.4 Å.

The Debye parameter, κ, is given by

$$\kappa = [8\pi\epsilon^2/(DkT)]^{1/2} I^{1/2} \qquad (6)$$

To facilitate comparison with the data of Wüthrich and coworkers for exchange rates of individual amide protons of BPTI in D_2O in the absence of added salt, we use a low value for the initial ionic strength, I_0 = 1 mM. At low and high pH, the added DCl or NaOD will make an additional contribution to the ionic strength. The ionic strength is approximated by

$$I = I_0 + [DCl] + [NaOD] = (1 \times 10^{-3}) + (10^{-pH}) + (10^{-(15.05-pH)}) \qquad (7)$$

Using T = 20 °C, and the dielectric constant for D_2O, w can now be calculated from eq 4 [at neutral pH (i.e., I = 1 mM), w = 0.238]. These values for w, and values for Z obtained with eq 1, are then used to calculate pH_{local} with eq 3. Finally,

[2] The 14 ionizable groups of BPTI (Wüthrich & Wagner, 1979) are grouped here into six categories on the basis of their pK values. These categories are as follows: two carboxylates with pK = 3.0, three carboxylates with pK = 3.7, the amino terminus with pK = 8.1, two tyrosines with pK = 10.1, five lysines with pK = 10.6, and two tyrosines with pK = 11.3. It was assumed that the six arginines remain protonated in the pH range considered (pH 0–12).

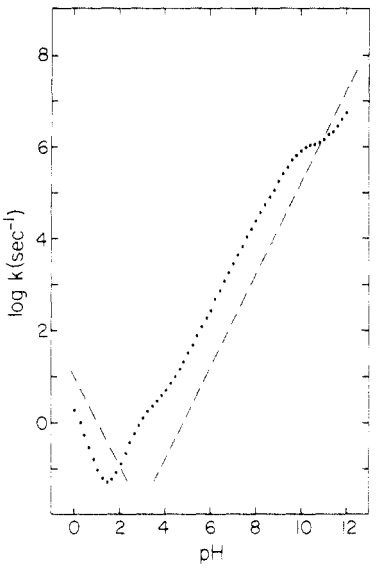

FIGURE 5: (···) Calculated pH dependence for the amide proton exchange of Tyr-35 using a smeared-charge model for BPTI and the nearest-neighbor parameters of Molday et al. (1972). Temperature = 20 °C and ionic strength = 1 mM. (---) Rates predicted by using only the nearest-neighbor parameters of Molday et al. (1972). See text for details of the calculations.

the rate of amide proton exchange, k_{HX}, at a given pH_{meas} is calculated by using pH_{local} rather than pH_{meas}. Appropriate values for the rate constants of acid- and base-catalyzed exchange are obtained by using the nearest-neighbor parameters of Molday et al. (1972).

The calculated pH dependence for the amide proton of Tyr-35 in BPTI at 20 °C is shown in Figure 5. Nearest-neighbor effects were evaluated with the parameters of Molday et al. (1972), which give the difference in rates between a specified amide proton and an Ala–Ala amide proton.[3] The rates for Ala–Ala were obtained from Englander et al. (1979). The results show a pH_{min} of 1.5, and the pH dependence curve is asymmetric. The slope (absolute value) of the pH dependence curve is sometimes greater than 1 (e.g., pH 0–1, pH 2–3) and sometimes less than 1 (e.g., pH 3–5, pH 9.5–11.5). When the electrostatic contributions to exchange are not included in these calculations but the same nearest-neighbor parameters are used, the predicted pH dependence is very different. If only nearest-neighbor effects are considered, then for Tyr-35 the pH_{min} is 2.9, and the slope (absolute value) of the pH dependence curve is uniformly equal to 1 except in the pH "minimum".

Discussion

Salt Effects on Amide Proton Exchange in N-Methylacetamide. The effect of salt on amide proton exchange has been studied for the model compound *N*-methylacetamide (NMA) by Schleich et al. (1968, 1971). Using NMR lineshape analysis, they observed salt effects on the amide proton exchange of NMA in both the acid- and the base-catalyzed regions of exchange. The magnitudes of these effects were dependent on the salt used. For NaCl, there was a 17% decrease in the acid-catalyzed exchange rate when 2.8 M NaCl was added to 1 M NMA (Schleich et al., 1968). The base-catalyzed exchange rate of NMA decreased 14% in the presence of 2.8 M NaCl (Schleich et al., 1971). Thus, the effect of NaCl on the amide proton exchange of the neutral species NMA is small.

Poly(DL-*alanine*). The effects of NaCl on the amide proton exchange of PDLA are also small as shown in Figure 1. Both the acid- and the base-catalyzed reactions are independent of NaCl concentration, within our experimental error. Englander & Poulsen (1969) also found no ionic strength dependence from 50 mM to 1.0 M for PDLA at pH 3, 0 °C.

Poly(DL-*lysine*). In contrast to the neutral species NMA and PDLA, exchange from PDLL shows a marked sensitivity to NaCl concentration. Since PDLL is a polyelectrolyte with a high charge density, the effects of counterion condensation must be considered. The theory for polyelectrolyte condensation has been developed by Manning (1978) and tested for DNA [reviewed by Record et al. (1978)]. In general, the theory predicts that a linear polyelectrolyte with a high charge density (e.g., PDLL or DNA) will be surrounded by a finite local concentration of counterions even when immersed in a solution in which the bulk counterion concentration is vanishingly small. Furthermore, the theory predicts that this local concentration of condensed counterions (C_{local}) will remain constant over a broad range of bulk salt concentration (C_{bulk}), provided $C_{bulk} < C_{local}$. The magnitude of C_{local} depends on the axial charge spacing [for PDLL = 3.64 Å (Pauling et al., 1951)], the valence of the counterion, temperature, and dielectric constant of the solvent. For PDLL in D_2O at 20 °C, we calculate that C_{local} = 0.25 M when a univalent counterion (e.g., Cl^-) is present. This represents a 50% charge neutralization of the lysine side chains by condensed Cl^- ions.

A small but significant fraction of the condensed counterions will be hydroxide ions. Thus, the pH in the vicinity of the PDLL molecule (pH_{local}) will be different from the measured pH of the bulk solution (pH_{meas}). The addition of NaCl to the solution will cause a decrease in pH_{local}, since the added Cl^- ions will compete with OH^- ions for the counterion layer around PDLL. A theory describing the competition between two counterions has been given by Manning (1978).

Therefore, NaCl influences the rate of amide proton exchange from PDLL in two ways: (i) As discussed above, the addition of NaCl will decrease the difference between pH_{meas} and pH_{local} for PDLL, since there is a competition between Cl^- and OH^- for the counterion layer surrounding PDLL. (ii) The addition of NaCl will also result in increased screening of the reactive species H_3O^+ and OH^- and of the positively charged PDLL (counterion condensation provides only 50% charge neutralization). Both of these salt effects (i.e., counterion competition and electrostatic screening) will enhance the rate of acid-catalyzed exchange and diminish the rate of base-catalyzed exchange for PDLL.

The effect of NaCl on the base-catalyzed exchange (Figure 2) is greater than on the acid–catalyzed exchange of PDLL (Figure 3) for two reasons. (i) The measurements of the acid-catalyzed exchange (Figure 2) were made at pH 1.23. At this pH there is a small but significant contribution of base catalysis at low salt concentrations (Figure 4). (ii) Even in the absence of added NaCl, the ionic strength at pH 1.23 is approximately 60 mM, owing to the DCl added to lower the pH. Figure 3 shows that, for the base-catalyzed reaction, the addition of 60 mM NaCl causes approximately a 10-fold decrease in the exchange rate.

General Base Catalysis. Klotz & Frank (1965) made the interesting observation that general acids and bases can catalyze amide proton exchange of NMA in dioxane–water

[3] The nearest-neighbor effects were evaluated by Molday et al. (1972) at moderately high ionic strengths (0.1 M KCl and 0.1–0.3 M peptide derivative). Thus, although local charge effects may be partly responsible for the observed differences in rates, the majority of the nearest-neighbor effects are likely to be inductive in nature and should therefore be included in these calculations.

mixtures, but in pure D_2O the effects are much smaller. Englander & Poulsen (1969) found that exchange from PDLA in H_2O is insensitive to general acids and bases (i.e., 1 M solutions of arginine, histidine, Tris, or trichloroacetate), and Molday et al. (1972) showed that 0.3 M lysine side chain does not serve as a general catalyst. Thus, it is unlikely that the lysine side chain is serving as a general base or as a general acid in catalyzing exchange from PDLL in D_2O. In any case, the effects of NaCl on the acid- and base-catalyzed reactions demonstrate that the effects of charge on the kinetics of exchange are important.

BPTI. In our calculations of the electrostatic effects on exchange from BPTI, we use a smeared-charge model (Linderstrøm-Lang, 1924; Tanford, 1962) to define a local pH. We then assume that the local pH determines the rate of amide proton exchange. Similar assumptions have been made previously (Coleman & Willumsen, 1969; Ikegami & Kono, 1967). The concept of local pH breaks down when one considers a discrete-charge model (Tanford & Kirkwood, 1957; Shire et al., 1975).

Our computed results (Figure 5) demonstrate that the electrostatic contribution to amide proton exchange in proteins may be very large. We predict that charge effects are important in interpreting data for BPTI collected in the absence of added salt (Richarz et al., 1979). The experimental data for Tyr-35 at 22 °C and our predicted curve (Figure 5) both show some anomalous features, by comparison with the pH dependence predicted solely from the nearest-neighbor parameters of Molday et al. (1972). The experimental pH_{min} is low (1.7 vs. 2.9 predicted from the nearest-neighbor parameters), and slopes both greater than 1 and less than 1 are observed experimentally in certain regions (Richarz et al., 1979). However, the overall agreement between the experimental curve and our predicted curve is poor. The experimental data of Richarz et al. (1979) and Wüthrich et al. (1980) differ from our predicted curves in important respects. Evidently factors in addition to net charge affect the pH dependence curve. From the analysis of protein titration curves by Shire et al. (1975), we would expect the local charge environment also to be an important factor.

Other anomalous features of the pH dependence curves for protons in BPTI (e.g., abnormally high pH_{min} values and also broad pH minima) have been discussed by Hilton et al. (1981). A "plateau" in the pH dependence curves for amide protons in BPTI, similar to our calculated plateau at pH 9.5–11.5 (Figure 5), has been observed by Hilton & Woodward (1979). However, their experiments were done in the presence of 0.3 M KCl, which will greatly reduce the electrostatic effects on amide proton exchange.

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