151.
Miller, J. H. (1972) *Experiments in Molecular Genetics,* p 432, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Offord, R. E. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M., Eds.) p 525, Oxford University Press, Oxford.
Payne, J. W. (1968) *J. Biol. Chem. 243,* 3395.
Payne, J. W. (1974) *J. Gen. Microbiol. 80,* 269.
Payne, J. W. (1976) *Adv. Microbiol. Physiol. 13,* 55.
Payne, J. W., & Gilvarg, C. (1968a) *J. Biol. Chem. 243,* 355.
Payne, J. W., & Gilvarg, C. (1968b) *J. Biol. Chem. 243,* 6291.
Payne, J. W., & Gilvarg, C. (1971) *Adv. Enzymol. 35,* 187.
Piperno, J. R., & Oxender, D. L. (1968) *J. Biol. Chem. 243,* 5914.
Ramos, S., & Kaback, H. R. (1977a) *Biochemistry 16,* 848.
Ramos, S., & Kaback, H. R. (1977b) *Biochemistry 16,* 854.
Rando, R. R. (1977) *Methods Enzymol. 46,* 28.
Roseman, S. (1972) in *The Molecular Basis of Biological Transport* (Woessner, J. F., & Huijing, F., Eds.) p 181, Academic Press, New York, N.Y.
Shankman, S., Higa, S., Florsheim, H. A., Schvo, Y., & Gold, V. (1960) *Arch. Biochem. Biophys. 86,* 204.
Shankman, S., Gold, V., Higa, S., & Squires, R. (1962) *Biochem. Biophys. Res. Commun. 9,* 25.
Silk, D. B. A. (1974) *Gut 15,* 494.
Simoni, R. D. (1972) in *Membrane Molecular Biology* (Fox, C. F., & Keith, A. D., Eds.) p 284, Sinaur Press, Stamford, Conn.
Simoni, R. D., & Postma, P. W. (1975) *Annu. Rev. Biochem. 44,* 523.
Singh, A., Thornton, E. R., & Westheimer, F. H. (1962) *J. Biol. Chem. 237,* PC3006.
Smith, P. A. S. (1970) in *Nitrenes* (Lwowski, W., Ed.) p 99, Wiley, New York, N.Y.
Staros, J. V., & Richards, F. M. (1974) *Biochemistry 13,* 2720.
Wold, F. (1977) *Methods Enzymol. 46,* 3.

Nuclear Magnetic Resonance Measurement of Hydrogen Exchange Kinetics of Single Protons in Basic Pancreatic Trypsin Inhibitor†

Bruce D. Hilton and Clare K. Woodward*

ABSTRACT: Hydrogen-deuterium exchange rates of single protons assigned to peptide amide NH have been measured in basic pancreatic trypsin inhibitor (BPTI) by proton nuclear magnetic resonance spectroscopy. The pD dependence of exchange rates for 8 slowly exchanging protons was measured over the range of pD 2.1–7.2. For the slowly exchanging BPTI protons, pD_{min}, the pD at minimum rate, $\simeq$ 4, while in random coil polypeptides, pD_{min} = 2.5–3. The exchange rates at pD_{min} are four orders of magnitude less than those calculated for the random conformation of BPTI. The observed exchange with respect to D^+ and OD^- ions is not first order, as expected from model compounds; there is a change of a factor of 2.5–4 in exchange rate per pD unit. Taken together, the features of the pH dependence suggest that the exchange event is not occurring in bulk solvent. This supports a mechanism for the rate-limiting protein conformational process(es) involving multiple small amplitude protein fluctuations that accommodate the penetration of the solvent species into the interior of the folded protein. The temperature dependence was measured at pD 4.6, 52–70 °C, and at pD 7.25, 58–68 °C, with the observable temperature range at a single pD limited by the high activation energies of 49–89 kcal/mol ± 10%. This suggests that in BPTI these very slowly exchanging protons are in regions of the structure in which small displacements of the surrounding atoms have high energy barriers, and that each proton has a different average set of protein motions determining its exchange. One of the downfield exchangeable resonances may not be a peptide NH as it does not exhibit the characteristic doublet splitting from $NH-C_\alpha H$ coupling. For 7 of the 10 resonances observed over this pD range, the chemical shift changes <0.05 ppm. Three resonances shift upfield 0.05–0.1 ppm as the pD is raised from pD 2 to 7, indicating the proximity of a titrating group, probably carboxyl. A marked increase in resolution is observed below pD 3, suggesting a decrease in rotational correlation time at the lower pD, perhaps from dissociation.

The exchange rates of protein peptide amide protons with solvent are a measure of the accessibility of solvent species to the polypeptide backbone. The hydrogen exchange kinetics of a folded protein are many orders of magnitude slower than those of the random conformation of the same protein, in keeping with the solvent shielding expected in a solution structure that approximates the crystal structure. However, the existence of finite exchange rates for the great majority of exchangeable protons necessitates the modification of models derived from the crystal structure of a rigid, tightly packed, solvent impenetrable protein, to include dynamic processes for the solution structure of folded proteins. In many proteins, 80–90% of the exchangable protons exchange within 24 h under conditions in which the folded conformation of the protein is greatly favored (Woodward & Rosenberg, 1971; Wickett et al., 1974; Woodward et al., 1975; Ellis et al., 1975).

The interpretation of hydrogen exchange kinetics in terms of the rate-limiting protein conformational process(es) involves

† From the Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108. *Received February 23, 1978.* This work is supported by National Institutes of Health Grants GM-18466 and HL-06314.

0006-2960/78/0417-3325$01.00/0

an analysis of the differences between the hydrogen exchange rates of native protein and those of its random conformation, for which there are extensive model compound data (Hvidt & Nielsen, 1966; Englander et al., 1972). The hydrogen exchange rates of native proteins as compared with those of a random coil are not only slowed, but also are distributed over an increased range. For example, at 10 °C, pH 3, the rate distribution of oxidized ribonuclease is $\simeq 10^{-1}$ to 10^{-3} min^{-1} (Woodward & Rosenberg, 1970) while that of native ribonuclease is broader than 10^{-1} to 10^{-5} min^{-1}, the limits of observation for standard hydrogen–tritium exchange experiments (Woodward & Rosenberg, 1971). Until recently, hydrogen isotope exchange techniques measured the exchange rates of all labile protons simultaneously, and the resulting complicated kinetics has hampered the analysis of hydrogen exchange phenomena. This difficulty can be overcome by the utilization of proton nuclear magnetic resonance (NMR) spectroscopy by which the exchange behavior of individual proton resonances with solvent deuterium can be studied.

Basic pancreatic trypsin inhibitor (BPTI)[1] offers many advantages for such a study. It is a small protein, molecular weight 6255, for which the crystal structure is known (Huber et al., 1971). Because of the unusual stability of BPTI, experiments may be carried out over a wide range of pHs and temperatures. The proton NMR spectrum of BPTI has $\cong 16$ down-shifted resonances individually resolved away from the main aromatic envelope. In addition to the description of this NMR spectral region downfield of the aromatic resonances, there have been extensive NMR studies of the aromatic protons of BPTI (Wagner & Wuthrich, 1975; Snyder et al., 1976). Also proton NMR studies of the methylene region (Brown et al., 1976) and ^{13}C NMR studies (Richarz & Wuthrich, 1977; Wuthrich & Bauman, 1976) have been reported for BPTI. Down-shifted exchangeable protons have also been reported in the proton NMR spectrum of other proteins (Patel & Canuel, 1976; Stellwagen & Shulman, 1973; Campbell et al., 1975).

Experimental Section

Bovine pancreatic trypsin inhibitor (BPTI) (Novo Industries, Denmark) and sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ (TSP) (Merck, Sharp & Dohme) were used without further purification. D_2O (99.8%) (Stohler) was treated by extraction with dithiocarbazone to remove metals in carbon tetrachloride (Hilton & Bryant, 1976).

BPTI, buffers, and solvents were preequilibrated to 25 °C for at least 1 h prior to use. Protein was added directly into deuterated solvents buffered with 0.02 M deuterated glycine, acetate, or phosphate and 0.3 M in KCl. Alternatively the protein was dissolved in 0.3 M KCl with pD adjusted with DCl. Buffer ions were interchanged without noticeable effects on the results. Spectra were obtained using a Bruker HX 270-MHz spectrometer equipped with a temperature control and 5-mm sample tubes. Temperatures were measured before and after experiments using ethylene glycol calibration (Piccinni-Leopardi et al., 1976); the variation in temperature was less than 0.3 °C. pH was measured as a Corning Model 112 pH meter using a glass electrode. pDs reported are the pH meter reading (at 68 °C) + 0.4 (Glasoe & Long, 1960).

NMR spectra were obtained in the Fourier transform mode using an internal deuterium lock. From 1500 to 10 800 scans were accumulated. Spectra were continuously recorded during an experiment while the sample remained in the spectrometer.

BPTI samples used for measurement of activation energy at pD 7.25 were 50 mg per 1.00 g of deuterated solvents; in all other cases 25 mg per 1.0 g of solvents was used.

Resonance intensities were obtained from the resonance area by a Gelman planimeter. The resonance assigned to the two exposed tyrosine-23 meta ring protons (Snyder et al., 1975) is a convenient internal area and chemical shift reference. The baseline was constructed by connecting minima between clearly resolved resonances, as in the pD 3.61 spectrum in Figure 1, which presented a difficulty for resonances 8, 9, 10, and 11 with the result that fewer rates are obtained for these (see Results). For resonances for which an objective choice of baseline cannot be made, the measurement of exchange rates is not attempted. In many cases data for slower exchanging resonances are obtained only after exchange of nearby resonances. This procedure is not theoretically exact, but gave reproducible linear semilogarithmic plots of resonance area vs. time with a minimum of decision making on the part of the operator. Attempts to analyze overlapping spectra mathematically did not give noticeably superior results.

In general the error in the measured exchange rates was estimated at less than ±10% with most of the error attributable to variation in temperature, since the apparent activation energies are quite high. The precision of individual rate constants based on a single exchange run was in general much better, usually ±5%. The standard deviations of the apparent activation energies reported are estimated to be about ±10% based on a linearized least-squares treatment of the data.

The fits of the pH-rate profiles to equation 2 were obtained by calculation of χ^2 for selected trial values of k_1, k_2, k_3, and X. In this way the amount of interaction between variables, and hence of the range of values of each variable allowed for reasonable fits to the data, could be estimated. Most significant is the fact that wide variations in the values of k_1 and k_2 were necessary for a rather narrow range of values for X in order to obtain reasonable values of χ^2. pOD was obtained from the measured pD using $pK_w = 13.58$, the value obtained by extrapolating from lower temperature measurements of K_w in D_2O (Weast, 1968).

Chemical shifts are relative to TSP. In general, shifts were first obtained relative to the tyrosine-23 resonance, measured to be 6.330 ppm in the pD range 2.15–7.25, and then related to the TSP standard.

Results

Figure 1 shows the spectra of the downfield region of the proton NMR spectrum of BPTI at pD 7.25–2.15 after approximately 1 h at 68 °C. The high-field region, from 6 to 7.5 ppm, consists of phenylalanine and tyrosine proton resonances; the low-field region 7.5–10.5 ppm consists of exchangeable protons. The aromatic region has been extensively characterized (Snyder et al., 1975; Wagner et al., 1976), and partial characterization of the exchangeable resonances at lower temperatures has also been reported (Masson & Wuthrich, 1973; Wagner & Wuthrich, 1976; Karplus et al., 1973; Marinetti et al., 1976). Each clearly resolved resonance in this region has an equivalent area when extrapolated to zero time and is equal to one proton by comparison with the tyrosine resonance at 6.33 ppm. These resonances have been observed to exhibit fine structure of doublet character (Karplus et al., 1973; Masson & Wuthrich, 1973) and the spectrum at pD 2.15, Figure 1, shows this feature clearly. The improved resolution of individual spectra at low pD, Figure 1, is not well understood (see Discussion). The chemical shifts of the exchangeable

[1] Abbreviations used: BPTI, basic pancreatic trypsin inhibitor; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; STI, soybean trypsin inhibitor.

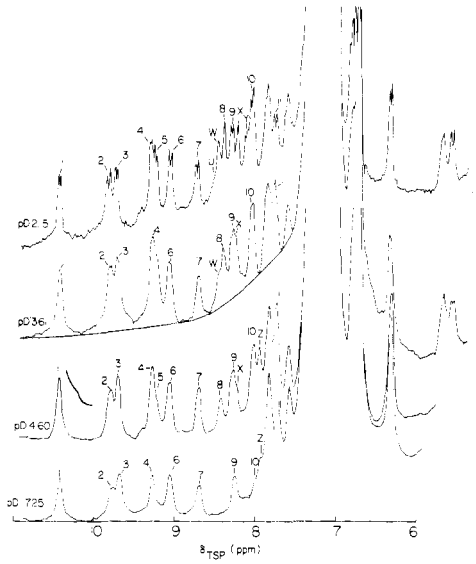

FIGURE 1: The proton NMR spectra of the downfield region of BPTI, obtained at 270 MHz after approximately 1 h at 68 °C. Exchangeable resonances observed through most of the pD range studied are numbered; other exchangable resonances are lettered. Lettered resonances are not observable over the entire pD range, as a result of increased base-catalyzed exchange rate far from the pD_{min}. Chemical shifts are ppm downfield from TSP; 10 800 transients were obtained for each spectrum. The samples at pD 2.15, pD 3.61, and pD 4.60 are 0.02 M in acetate and 0.3 M in KCl; the sample at pD 7.25 is 0.02 M in phosphate and 0.3 M in KCl.

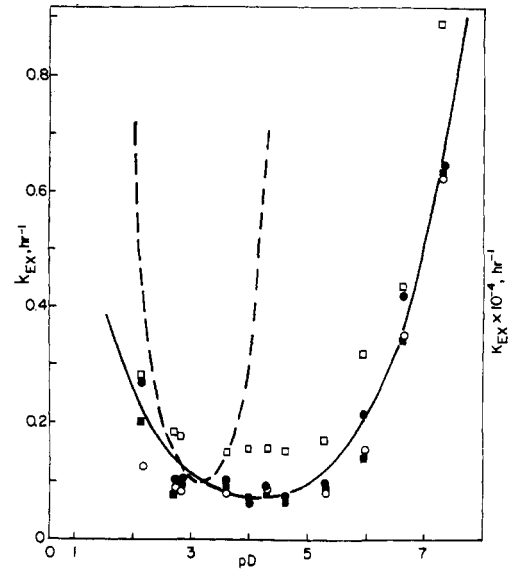

FIGURE 2: pD dependence of the exchange rates of resonances 1 (●), 2 (□), 3 (■), and 4 (○) at 68 °C. The theoretical curve was calculated for a fit to the data for resonance 1 to eq 2, with $k_1 = 1.4\ h^{-1}\ M^{-0.4}$, $k_2 = 245\ h^{-1}\ M^{-0.4}$, $k_3 = 0$, and $x = 0.4$; pOD was calculated using $pK_w = 13.58$. The dashed line (see units on right) represents the experimental curve for poly(DL-alanine) (Englander et al., 1972): $t_{1/2} = 200/(10^{pH-3} + 10^{3-pH})(10^{0.05T})$, where T is in °C and $t_{1/2}$ is the half-time for exchange in minutes. The pH was set equal to pD; otherwise, no correction was made in the above equation.

resonances, with the exception of 7, 9, and 11, were observed to be constant within 0.05 ppm at pDs between 2.15 and 7.25 and over the temperature range of these experiments. Resonances 7, 9, and 11 show an apparent shift of 0.05–0.1 ppm downfield with decreasing pD between pD 7 and 2.

By suitable choice of pD and temperature, it is possible to measure the exchange rates of these resonances through repeated scanning of the downfield region as a function of time. In all cases, the decay of the resonance area, measured over 1.5–2 decades of time, is characterized by a single exponential time constant.

Figures 2 and 3 show the measured exchange rates as a function of pD. Resonance numbering corresponds to that in Figure 1. The exchange properties of the pH dependent resonances aid the numbering of these at different pDs. Differences in the number of exchangeable resonances observed in this report and those of previous reports may be attributed to differences in temperature; at high temperatures some resonances exchange too rapidly to be observed.

The temperature dependence was also measured. Due to the high activation energies, only a narrow temperature window is available. Exchange rates vs. temperature were obtained at pD 7.25, Table I, and at pD 4.60, Table II. The apparent activation energies for those resonances for which reliable rates could be obtained over the entire temperature range are reported in Table III.

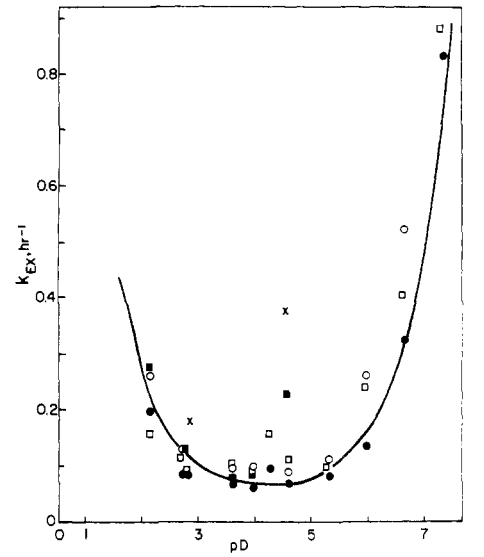

FIGURE 3: pD dependence of the exchange rates of resonances 6 (●), 7 (□), 8 (X), 9 (○), 10 (■) at 68 °C. The solid line was calculated for a fit to the data for resonance 6 to eq 2, with $k_1 = 2.7\ h^{-1}\ M^{-0.6}$, $k_2 = 5200\ h^{-1}\ M^{-0.6}$, and $k_3 = 0.035\ h^{-1}$, and $x = 0.6$; pOD was calculated as in Figure 2.

Discussion

The downfield exchangeable resonances of BPTI between 7.5 and 11 ppm, numbered 1–11 in Figure 1, are assigned to peptide NH protons based on their very slow exchange rates, their relatively large downfield chemical shifts which are readily explained by intramolecular hydrogen bonding, and their doublet splitting that would result from NH-C_αH coupling (Masson & Wuthrich, 1973; Karplus et al., 1973). It has been noted that the doublet splitting could as well arise from immobilized NH_2 groups in which the nonequivalent NH_2 protons couple to each other (Karplus et al., 1973), but the number of resonances involved is large, precluding the possibility that all of them may arise from amino acid side-chain protons.

The exchange rates of these protons are extraordinarily slow, with half times of >4 months at ambient temperature and neutral pH (Karplus et al., 1973). To obtain exchange rates observable within 24 h, these experiments are carried out at 50–70 °C, above the thermal unfolding temperature for most globular, soluble proteins, but not for BPTI (Vincent et al., 1971; Masson & Wuthrich, 1973; Wagner et al., 1976).

The difference between the pH dependence of the exchange

TABLE I: Rate Constants for Out-Exchange of Downfield Resonances of BPTI, pD 7.25, at Varying Temperatures.

resonance	exchange rates[a] (h^{-1}) 58.4 °C	59.7 °C	62.3 °C	65.0 °C	68.0 °C
1	0.042	0.093	0.099	0.217	0.645
2	0.105	0.169	0.213	0.294	0.893
3	0.038	0.075	0.087	0.200	0.549
4	0.064	0.119	0.154	0.192	0.649
6	0.026	0.065	0.088	0.270	0.645
7	0.055	0.116	0.141	0.256	0.833
9	0.061	0.123	0.128	0.357	0.877

[a] Rates are within ±10% (see Experimental Section).

TABLE II: Rate Constants for Out-Exchange of Downfield Resonances of BPTI, pD 4.60, at Varying Temperatures.

resonance	exchange rates[a] (h^{-1}) 62.3 °C	65.3 °C	66.6 °C	69.4 °C	70.7 °C
1	0.020	0.079	0.075	0.160	0.274
2	0.029	0.187	0.152	0.236	0.364
3	0.011	0.088	0.060	0.135	0.239
4	0.013	0.102	0.066	0.145	0.250
6	0.011	0.061	0.068	0.170	0.216
7	0.019	0.100	0.113	0.340	
9		0.070	0.089	0.179	

[a] Rates are within ±10% (see Experimental Section).

rates of the downfield resonances of BPTI, Figures 2 and 3, and that of model compounds reflects the contribution from protein conformation to exchange from the native protein. For amides or primary amines the outstanding feature of a profile of pH vs. NH exchange rate is a minimum in the pH 3–5 region (Hvidt & Nielsen, 1966), the result of the NH exchange mechanism which is both acid and base catalyzed (Berger et al., 1959; Klotz & Frank, 1965).

The NH proton chemical exchange rate of model amides, k_{cx}, is given by

$$k_{cx} = k_H[H^+] + k_{OH}[OH^-] + k_0 \quad (1)$$

where k_H, k_{OH}, and k_0 are the apparent first-order rate constants for acid-catalyzed, base-catalyzed, and direct exchange with water, respectively (Berger et al., 1959). In model peptides freely exposed to the solvent, k_0 is negligibly small (Leichtling & Klotz, 1966; Berger et al., 1959). At the pH of minimum rate, pH_{min}, the first two terms of eq 1 are equal. The rate at pH_{min} is called k_{min}.

The data in Figures 2 and 3 show the first observation of the pD dependence of individual peptide NH protons in folded proteins over a pD range that spans the pD_{min}. The principal features of interest are the pD_{min}, the k_{min}, and the pD dependence in the acid- and base-catalyzed regions.

The pD–rate profiles of the slowly exchanging BPTI protons, Figures 2 and 3, show a remarkable similarity, each having approximately the same pD_{min}, k_{min}, and an unusual flatness, as compared with model compounds, in the region of the pD_{min}. The $pD_{min} \cong 4$ for all of the protons except possibly for resonances 8 and 10, for which less data has been obtained. The region of the pD_{min} is considerably shallower than that of model polypeptides, e.g., poly(DL-alanine) (Englander & Poulsen, 1969), the dashed curve in Figure 2. For poly(alanine), $pH_{min} \simeq 3$ (Englander & Poulsen, 1969); for *N*-methylacetamide, $pH_{min} \cong 5$ (Klotz & Frank, 1965; Leichtling & Klotz, 1966; Molday et al., 1972); for side-chain NH_2 protons,

TABLE III: Activation Energies and pH Parameters for Exchangeable BPTI Resonances.

resonance	E^*_{app} (kcal/mol)[a] pD 7.25	pD 4.60	pD_{min}[b]	k_{min} (h^{-1})[c]
1	59 ± 7	65 ± 6	4.1	0.07
2	49 ± 8	51 ± 12	4.1	0.15
3	59 ± 5	72 ± 13	4.0	0.06
4	52 ± 7	76 ± 13	3.7	0.06
6	72 ± 6	89 ± 8	4.2	0.05
7	58 ± 6	84 ± 8	4.0	0.09
9	64 ± 6		3.6	0.09
10			3.5	0.05

[a] Activation energies were obtained over the interval 58.4–68 °C at pH 7.25, and over the interval 62.3–70.7 °C at pD 4.60. The error, σ, is estimated from $\sigma^2 = \Sigma(y - a - bx)^2/N\Sigma x^2 - (\Sigma x)^2$, where y = rate (h^{-1}), $x = 1000/T$ (K), and a and b are linearized least-squares fit parameters. [b] The pD at minimum rate, pD_{min}, is obtained from the best fit of the data in Figures 2 and 3 to eq 2. [c] k_{min} is the value of k_{ex} at pD_{min}.

$pH_{min} \cong 4.5$ (Molday et al., 1972). The difference in pH_{min} for the free amide and the polypeptide can be accounted for by the inductive effects of neighboring peptide and side-chain groups (Molday et al., 1972). In general, an electron withdrawing group decreases the acid-catalyzed exchange rate and increases the base-catalyzed exchange rate and, hence, lowers the pH_{min} (Leichtling & Klotz, 1966).

From model compounds, one expects that the pH_{min} of the chemical exchange rate of different peptide NH protons will be similar, but somewhat different from each other due to the side chain substituent effects (Woodward & Rosenberg, 1970; Molday et al., 1972). Molday et al. (1972) have formulated a set of empirical rules by which the chemical exchange rates of each peptide amide of a protein can be calculated from its amino acid sequence. These are the exchange rates expected for the random chain conformation of the protein, in which the NH groups are freely exposed to solvent and have no contribution from protein folding to the exchange rates. The pH_{min} and k_{min} for the chemical exchange rates, at 68 °C, of the BPTI peptide and amide side-chain protons are listed in Table IV. The observed pD_{min}s $\cong 4$, Table III, are $\simeq 1.5$ pD units higher than those estimated for the freely exposed peptide protons, and about the same as those for amide side chains, Table IV.

It is unlikely, however, that the slowly exchanging BPTI protons arise from side chains because of the number observed. For all of the resonances with $pD_{min} \cong 4$ to be due to side chain NHs, each of the three asparagines and the glutamine would have to be immobilized at 58–70 °C, temperatures at which the buried aromatic side chains are rapidly rotating (Wagner et al., 1976).

It is more likely that these are peptide NH resonances and that the shift to higher pD_{min} is due to the relatively apolar environment of the exchanging proton. In an apolar solvent, pD_{min} is shifted to a higher value as compared with that in water (Leichtling & Klotz, 1966). For simple amides, an upward shift of pD_{min} by 1.5 units would reflect a shift of pK_w of plus $\cong 3$ units (Leichtling & Klotz, 1966). It is possible that, if k_H were to have a higher activation energy than k_{OH}, the pD_{min} would be shifted up at elevated temperatures. For poly(DL-alanine), the opposite is true; activation energies measured from 0 to 15 °C are slightly larger for k_{OH} (17 kcal/mol) as compared with k_H (15 kcal/mol) (Englander & Poulsen, 1969).

Because pD_{min} for different protons varies, for consideration

TABLE IV: Values of pH_{min} and k_{min} Calculated for Individual Residues in the Random Coil Conformation of BPTI.

residue[a]	pH_{min}[b]	k_{min}[b] (h^{-1})	residue[a]	pH_{min}[b]	k_{min}[b] (h^{-1})	residue[a]	pH_{min}[b]	k_{min}[b] (h^{-1})	residue[a]	pH_{min}[b]	k_{min}[b] (h^{-1})
Arg-1			Ala-16	2.7	622	Gln-31	2.4	1108	Lys-46	2.6	658
Pro-2	1.0	622	Arg-17	2.65	937	Thr-32	2.45	835	Ser-47	2.3	524
Asp-3	3.3	1766	Ile-18	2.7	622	Phe-33	2.35	938	Ala-48	2.55	1180
Phe-4	2.7	590	Ile-19	3.0	1045	Val-34	2.95	744	Glu-49	2.7	2611
Cys-5	2.6	622	Arg-20	2.65	937	Tyr-35	2.8	829	Asp-50	3.4	2487
Leu-6	2.55	1180	Tyr-21	2.5	495	Gly-36	2.9	1243	Cys-51	2.5	613
Glu-7	2.7	2611	Phe-22	2.75	592	Gly-37	2.65	2641	Met-52	2.55	1180
Pro-8	3.1	1554	Tyr-23	2.75	592	Cys-38	2.35	1324	Arg-53	2.6	937
Pro-9	3.0	1045	Asn-24	2.7	1552	Arg-39	2.2	1045	Thr-54	2.3	524
Tyr-10	2.8	829	Ala-25	2.8	1655	Ala-40	2.7	622	Cys-55	2.2	989
Thr-11	2.6	622	Lys-26	2.65	937	Lys-41	2.65	937	Gly-56	2.5	1971
Gly-12	2.5	1971	Ala-27	2.7	622	Arg-42	2.3	556	Gly-57	2.65	2641
Pro-13	2.7	1565	Gly-28	2.9	1757	Asn-43	2.4	1178	Ala-58	3.45	351
Cys-14	2.6	880	Leu-29	2.7	1565	Asn-44	2.55	2641	side chains		
Lys-15	2.2	1045	Cys-30	2.6	880	Phe-45	2.6	1315	Asn	3.9	4.05×10^4
									Gln	4.2	2.03×10^4

[a] In the x-ray structure residues 3–7, 48–56 are assigned to an α-helix structure, and residues 15, 16, 18–24, 30–37, and 44–46 are assigned to a β-sheet structure (Levitt & Greer, 1977). [b] pH_{min} and k_{min} are calculated for k_{ex}, the rate constant for chemical exchange, from the rules of Molday et al. (1972) by use of a program developed by Dr. Lynda Ellis. In these calculations, the acid dissociation constant of water, K_w, = 14. Chemical exchange rates at 68 °C are calculated from data obtained at 25 °C (Molday et al., 1972) and using the empirical equation for temperature dependence of Englander & Poulsen (1969) which was determined over the temperature range 0–15 °C.

of protein conformational contributions to exchange rates, k_{min}, rather than the observed exchange rate, k_{ex}, at an arbitrary pH, is more meaningful for the comparison of the model compound chemical exchange rates with those of folded proteins.

A decrease in k_{min} can arise from steric shielding factors, from changes in the nature of the solvent microenvironment, as well as from the effects of intramolecular hydrogen bond participation. There has been a tendency (Englander & Staley, 1969; Welch & Fasman, 1974; Makanishi & Tsuboi, 1974) to equate the number of protons exchanging more slowly to folded proteins or structured polypeptides than in the random chain conformation to the number of intramolecular hydrogen bonded protons. We have argued (Woodward et al., 1975; Ellis et al., 1975; Woodward & Ellis, 1975) that such a physical interpretation of the first-order terms fit to complex kinetics is not warranted and that the vastly decreased values and increased distribution of exchange rates in folded proteins and structured polypeptides are due to the restriction of solvent accessibility of protons that may or may not be intramolecularly hydrogen bonded. There are several examples of amide NH protons unable to form hydrogen bonds but having greatly slowed exchange rates (Scarpa et al., 1967; Brewster & Bovey, 1971). Steric factors that decrease the accessibility of OH^-, H_3O^+, and H_2O will decrease all three terms on the right of eq 1. The nature of the solvent environment affects k_{min} such that k_{min} drops as pK_w increases. Also, for the model compound, *N*-methylacetamide, at 25 °C, a drop in the D^+ concentration at pD_{min} results in a decrease of k_{min} by approximately the same factor. Thus, for model compounds, one would expect that a shift of pD_{min} by +1.5 units would result in a decrease of k_{min} by a factor of $\simeq$30 (Leichtling & Klotz, 1966).

As the pH is raised or lowered from pH_{min}, the base- or acid-catalyzed reaction dominates the exchange process. In the linear regions of the pH–rate profile a change in rate of a factor of 10 per pH unit is expected for simple acid or base catalysis, as observed for amides and polyalanine (Englander et al., 1972). However, the pD dependence in the acid- and base-catalyzed arms of the pD profile of these BPTI protons shows a relative change in rate of a factor of 2.5–4 per pH unit; i.e., the exchange rate in the base-catalyzed region is apparently not first order with respect to (OD^-) in the bulk solution. The observed exchange rate, k_{ex}, can be fit with an equation of the form

$$k_{ex} = k_1[D^+]_b{}^x + k_2[OD^-]_b{}^x + k_3 \qquad (2)$$

where b refers to the bulk solvent ion concentrations, and k_1, k_2, and k_3 are constants. The exponential data of Figures 2 and 3, with the exceptions of resonances 8 and 10 for which there are only fragmentary data, cannot be fit with an equation of the form of eq 2 with values of $x > 0.6$. Reasonable fits gave values of x between 0.4 and 0.6. Although our data do not extend very far into the linear region of the hydroxyl ion catalyzed region since the rates at higher pH become too fast to measure at 68 °C, it is clear that values of x are well below 1, and that the shape of the pD–rate curve is not due to the effect of pD independent exchange. The shallowness of the fitted pD vs. rate curve of Figures 2 and 3, as compared with those of model compounds, is reflected in a value of $x < 1$, with no contribution from a pD independent term except possibly for resonance 2.

Resonances 1–5, 8, and 10, Figure 1, do not vary over 0.05 ppm in chemical shift between pD 2.1 and 7.2. Resonances 7, 9, and 11 show more marked pD dependent chemical shifts, shifting upfield 0.05–0.1 ppm as the pD is raised from pD 2.1 to 7.2. In model peptides from pH 1 to 5, only the C-terminal peptide NH experiences pronounced titration shifts of $\cong$0.4 ppm, while peptide protons of aspartic, glutamic and, possibly, histidine residues in the polypeptide chain show shifts $\leq$0.05 ppm (Bundi & Wuthrich, 1977). The pD dependence of the chemical shifts of at least two of the resonances 7, 9, and 11 must be due to titration of carboxyl groups in three-dimensional proximity.

Resonances 1–7, 9–11, and y, Figure 1, have clear doublet splitting, but resonances 8, w, and x are not split, suggesting that the latter three resonances do not arise from peptide amide protons but rather from other types of exchangeable protons.

There is a pronounced pD-dependent feature of the NMR spectra of the downfield region of BPTI, namely, that, below pD 3, the resonances show considerably higher resolution, e.g.,

Figure 1. This could be due to a dissociation from a dimer to a monomer form of the protein at low pD. There is evidence that BPTI is a dimer around neutrality and room temperature (Kraut et al., 1960; Sholtan & Lie, 1966), but no information is available for aggregation at these temperatures.

In summary, the pD dependence of exchange of the BPTI downfield resonances 1–4, 6, 7, and 9, Figure 1, as compared with that of model compounds, shows that the exchange rates go through a pD_{min} indicating that D_3O^+, OD^-, and D_2O are present at the exchange site and exchange involves a charged intermediate, that the pD–rate profile is considerably more shallow for the BPTI protons, that pD_{min} of the BPTI protons is shifted up to $\cong$1.5 units, that the D^+ and OD^- dependence of the BPTI protons away from the pD_{min} is much less than first order, and that at 68 °C exchange of these BPTI protons is limited by conformational processes that slow it by $\cong$4 orders of magnitude as compared with the chemical exchange step. Also, the chemical shifts of resonances 7, 9, and 11, Figure 1, are affected by the titration of a carboxyl group(s), resonance 8 may not be a peptide amide proton resonance, and the decreased line width at low pH suggests a decrease in rotational correlation time of BPTI below pD 3.

The temperature dependence of the slowly exchanging BPTI protons between 58–71 °C gives a high activation energy of $\cong$50–90 kcal/mol, Table III, as compared with $\cong$20 kcal/mol for model polypeptides (Englander et al., 1972). In tritium–hydrogen exchange studies of ribonuclease, trypsin, lysozyme, STI, chymotrypsinogen, and carbonic anhydrase in which all of the NH exchange rates are measured simultaneously, activation energies have been estimated from the ratio of exchange times at a constant number of protons remaining unexchanged (Woodward & Rosenberg, 1971; Woodward et al., 1975; Wickett et al., 1974; Ellis et al., 1975; Rosenberg & Enberg, 1969; Rosenberg & Chakravarti, 1968). Activation energies obtained by this procedure are 20–35 kcal/mol for 80–90% of the measurable exchanging protons at pH around neutrality. The exchange rates of the slowest exchanging protons in these proteins can be measured only at elevated temperatures, above $\cong$ 35–40 °C, when there is a steep rise in activation energies and a marked decrease in the distribution of exchange rates. The high activation energies and decreased distribution of exchange rates at high temperatures for ribonuclease, trypsin, and STI are accounted for by contributions to the exchange process from thermal unfolding (Woodward & Rosenberg, 1971; Woodward et al., 1974; Ellis et al., 1975).

However, in BPTI, the high activation energies of slowly exchanging amide protons are not due to thermal unfolding, but to activation barriers to protein conformational processes in the folded macrostate of the protein that provide accessibility of OD^-, D_3O^+, and D_2O, to labile protons buried in the protein matrix.

Studies of H–D exchange monitored by NMR in BPTI (Hvidt & Pederson, 1976; Wagner & Wuthrich, 1976), and in other proteins (Crespi et al., 1974; Campbell et al., 1975; Patel & Canuel, 1976) have been reported. The temperature and pH dependence of single proton exchange rates is available only for one resonance in cytochrome *c* over a limited range of temperature and pH (Patel & Canuel, 1974). From these data (patel & Canuel, 1974) we calculate for the single resonance in cytochrome *c* an activation energy of $\cong$46 kcal/mol, 45–65 °C, pH 7; the pH dependence between pH 6.5 and 8, 55 °C, corresponds to a rate change of a factor of $\cong$7 per pH unit.

In addition to hydrogen isotope exchange phenomena, the accessibility of solvent species to buried regions of the folded conformation of globular proteins has been demonstrated by quenching of tryptophan fluorescence and phosphorescence (Lakowicz & Weber, 1973; Weber, 1975; Eftink & Ghiron, 1977; Savioti & Galley, 1974).

Models for protein conformational fluctuations responsible for solvent accessibility in folded proteins are of two basic types. One is characterized by large amplitude deviations in structural parameters from the folded conformation, with exchange occurring from an unfolded form. The second type involves many independent, low energy, small amplitude events, each too small to provide access to solvent by itself, but with a finite probability of collectively accommodating the penetration of water molecules and solvent ions into the interior of the protein where the exchange takes place. The first type of protein motional process may be equivalent to the major unfolding transition even at solution conditions at which the equilibrium greatly favors the folded form (Cooper, 1976). The first type of high amplitude process has also been suggested to be one of reversible, localized unfolding of segments of the protein to partially denatured forms (Englander, 1975; Schreier & Baldwin, 1976).

There are several types of models for the second type of conformational process involving many independent small amplitude motions (Lumry & Rosenberg, 1975; Weber, 1975; Careri, 1974; Careri et al., 1975; McCammon et al., 1977; Gō, 1976).

One of these (McCammon et al., 1977) is applied to BPTI and suggests that the internal regions of folded proteins are fluid-like at room temperature, and that structural variation is due to fluctuations confined to the neighborhood of the average structure, similar but not identical with the x-ray structure. Small amplitude internal motion in BPTI is demonstrated in the NMR spectral properties of the ring protons of the tyrosine and phenylalanine residues of BPTI, indicating rapid rotation of the aromatic rings in the folded structure (Snyder et al., 1975, 1976; Wagner et al., 1976; Wagner & Wuthrich, 1975) accommodated by small, low energy displacements of atoms surrounding the side chain (Gelin & Karplus, 1975; Hetzel et al., 1976).

One difference between the two types of models for the conformational process responsible for the slowed exchange rates in proteins is that, in the first, the exchange event occurs in bulk solvent, while in the second the exchange event occurs in the closely packed environment of the interior of the folded protein. The dependence of exchange rate on a bulk solvent property, pD, may be used to differentiate between these two types of protein internal motion. The pD dependence of the slowest exchanging BPTI protons is very different from that of small amides and unfolded polypeptides. First, the pD_{min} suggests that the exchange occurs in an apolar environment (see above). Secondly, there is a substantially less than first-order dependence on solvent OD^- ion concentration in the linear base-catalyzed region for the BPTI protons. A smaller deviation from the expected first-order dependence on bulk solvent hydroxyl ion concentrations has been reported for the intermediate NH exchange rates of several other proteins (Willumsen, 1971; Woodward & Rosenberg, 1971; Patel & Canuel, 1976), although a number of proteins do apparently show first-order dependence on hydroxyl ion concentration (Willumsen, 1971). The deviation from the expected pH dependence of hydrogen exchange in some proteins has been variously explained as an electrostatic effect (Coleman & Willumsen, 1969), or as due to the pH dependence of a protein conformational process, such as "breathing equilibria" (Englander et al., 1972). Neither of these can be full explanations of the observed pH dependence of Figures 2 and 3.

An electrostatic correction factor apparently accounts for the pH dependence of insulin below the isoelectric point (Coleman & Willumsen, 1969). A reasonable estimate of the pH dependence of such an electrostatic correction for BPTI exchange over the range pH 1–7 gives only a small fraction of the correction needed to explain the observed pH dependence.

Any pD dependent protein conformational process proposed to account for the differences between the profile of Figures 2 and 3, and that of model compounds must be one of two types, one in which k_{cx} does not enter the overall rate expression with all the pD dependence residing in the conformational reaction, or one in which k_{cx} and the conformational change both contribute to the overall pD dependence. For either type, complicated sets of pD dependent behavior must be invoked; for example, in the first type, the conformational transition would be pD independent in the region of the pD_{min}, the pD range over which normal carboxyls titrate, but show both a monotonic change with pD at pD >5 where there are no normally titrating groups in BPTI, and a reversal at pD <2.8. In the second type, the pH dependence of the conformational change that could compensate that of k_{cx} to give the observed rate profiles would go through a complex reversal to account for the shallowness and the symmetrical acid-catalyzed arm of the pD–rate profile. A pH-dependent monomer–dimer equilibrium could also make a contribution.

There is then, no likely, adequate explanation of how the exchange event can be taking place while the NH group is freely exposed to solvent and also exhibit the pD dependence in Figures 2 and 3. This suggests that the exchange event does not take place in bulk solvent, and therefore that solvent species penetrate, presumably by means of small amplitude fluctuations, into the matrix of the folded protein where exchange occurs. This interpretation is in keeping with that of the exchange behavior of protons at the interface of the trypsin–soybean trypsin inhibitor (Woodward, 1977; Woodward & Ellis, 1975), and with the viscosity dependence of acrylamide quenching of tryptophan fluorescence (Eftink & Ghiron, 1977).

We suggest that the pD dependence of exchange arises from the relationship of bulk solvent pD and the activities of OD^- and D^+ ions penetrating to the exchange site. The activity coefficients of one or a few (hydrated) OD^- or D^+ ions in a closely packed matrix is expected to deviate substantially from that in dilute aqueous buffer. The concentrations of solvent species that it is necessary to postulate as "inside" the protein in order to account for the hydrogen exchange data cannot, at present, be calculated directly from fits of the form of eq 1 because the activities of the OD^- and D^+ as a function of pD are not known. However, from simple considerations of concentration, the ratio of protein molecules to D^+ or OD^- ions is $1:\ll 1$ and there will probably be only one OD^- or D^+ ion present per exchanging protein molecule.

If the rate-limiting process for exchange involves small amplitude fluctuations which permit penetration of solvent into the folded protein, one must explain why such process(es) have high activation energies in this case but lower activation energies for several other proteins. Large values of E^* are usually taken as indicative of large amplitude fluctuations, as in thermal unfolding. We suggest, however, that small atomic displacements in tightly packed regions of the protein molecule may have higher energy barriers as compared with small motions in regions of lower packing density. An alternative suggestion is that most of the pD dependence arises from a low-energy process and small contributions from a second high-energy process account for the high values of E^* (Carter et al., 1978). Given the small range of temperatures, any interpretation of the apparent E^* must be tentative. The apparent shift to higher activation energies at lower pD, Table III, is not understood.

It is striking that not only pD_{min}, but also k_{min} values, are approximately the same for 7 of these exchanging protons, given the fact that exchange rates in the folded protein are slowed over a range of $\simeq 4$ orders of magnitude, as compared with the intrinsic exchange rates in Table IV. For all but resonance 2, k_{min} values are within a factor of 1.5 of each other, while the value of k_{min} for the chemical exchange varies over a factor of $\cong 5$, Table IV. This could be taken as a sign of a single conformational event, presumably large amplitude, which simultaneously exposes all of the protons to solvent. If this were the case one must then explain the pD dependence of such a conformational transition. Additionally, at lower temperatures, the range of k_{min} values will be much broader, corresponding to the range of activation energies, assuming these are constant with temperature. While our confidence in the values of E^* is limited by the very small temperature interval over which the rates can be measured at these pHs, the differences between the E^* values for several protons are outside experimental error, Table III. This suggests that there is more than one conformational process leading to accessibility for these protons.

Assignment of the NH resonances to specific amino acids will facilitate quantitative interpretation of the differences between the observed pD and temperature dependence of exchange in native BPTI and those of the intrinsic chemical exchange step derived from model compounds. While detailed assignments are not available, some narrowing down has been done.

Wuthrich and co-workers have assigned these downfield proton resonances to the hydrogen bonded protons in the β sheet from residues 16 to 36, and the α helix formed by residues 47 to 56 (Wagner & Wuthrich, 1976; Wuthrich et al., 1976).

Marinetti et al. (1976) have reported evidence from Gd(III)-induced relaxation experiments that resonances 1, 3, 4, 6, 7 and one resonance at higher field than 8.5 ppm (our numbering) are compatible with backbone protons in the β-sheet sequences 19–22 and 31–33. Refer to Table IV for the BPTI sequence. According to Levitt & Greer (1977), the hydrogen-bonded peptide NHs in these sequences are 20, 21, 22, 31, and 33. Resonance 2 is found to be compatible with protons of residues 12, 14, and 39 (Marinetti et al., 1976), none of which are hydrogen bonded by the criteria of Levitt & Greer (1977). Compared with the other resonances below 8.5 ppm, different relaxation behavior is observed for resonance 2 (Marinetti et al., 1976). It is interesting that resonance 2 also has a significantly different value of k_{min} compared with all the other exchangeable protons measured in this study, Figure 2.

Acknowledgment

The NMR spectra used for this article were obtained with the technical assistance of Mr. Robert L. Thrift, at the Freshwater Biological Institute, Navarre, Minnesota.

References

Berger, A., Loewenstein, A., & Meiboom, S. (1959) *J. Am. Chem. Soc. 81,* 62.

Brewster, A., & Bovey, F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 1199.

Brown, L., DeMarco, A., Wagner, G., & Wuthrich, K. (1976)

Eur. J. Biochem. 62, 103.
Bundi, A., & Wuthrich, K. (1977) *FEBS Lett 77,* 11.
Campbell, I., Dobson, C., & Williams, R. J. P. (1975) *Proc. R. Soc. London, Ser. B 189,* 485.
Careri, G. (1974) *Quantum Statistical Mechanics in the Natural Sciences,* p 15, Plenum Press, New York, N.Y.
Careri, G., Fasella, P., & Gratton, E. (1975) *Crit. Rev. Biochem. 3,* 141.
Carter, J., Knox, D., & Rosenberg, A. (1978) *J. Biol. Chem. 253,* 1947.
Coleman, D., & Willumsen, L. (1969) *C. R. Trav. Lab. Carlsberg 39,* 1.
Cooper, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2740.
Crespi, H., Kostka, A., & Smith, U. (1974) *Biochem. Biophys. Res. Commun. 61,* 1407.
Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16,* 5546.
Ellis, L., Bloomfield, V., & Woodward, C. (1975) *Biochemistry 14,* 4313.
Englander, S. (1975) *Ann. N.Y. Acad. Sci. 244,* 10.
Englander, S., & Poulsen, A. (1969) *Biopolymers 7,* 379.
Englander, S., & Staley, R. (1969) *J. Mol. Biol. 45,* 277.
Englander, S., Downer, N., & Teitelbaum, H. (1972) *Adv. Protein Chem. 21,* 810.
Gelin, B., & Karplus, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 2002.
Glasoe, P., & Long, F. (1960) *J. Phys. Chem. 64,* 188.
Gō, N. (1976) *Adv. Biophys. 9,* 65.
Hetzel, R., Wuthrich, K., Deisenhofer, J., & Huber, R. (1976) *Biophys. Struct. Mech. 2,* 159.
Hilton, B., & Bryant, R. (1976) *J. Magn. Reson. 21,* 105.
Huber, R., Kukla, D., Ruhlman, A., & Steigemann, W. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36,* 141.
Hvidt, A., & Nielsen, S. (1966) *Adv. Protein Chem. 21,* 287.
Hvidt, A., & Pederson, E. (1974) *Eur. J. Biochem. 48,* 333.
Karplus, S., Snyder, G., & Sykes, B. (1973) *Biochemistry 12,* 1323.
Klotz, I., & Frank, B. (1965) *J. Am. Chem. Soc. 87,* 2721.
Klotz, I., & Mueller, D. (1969) *Biochemistry 8,* 12.
Kraut, H., Körbel, W., Scholtan, W., & Schultz, F. (1960) *Hoppe-Seyler's Z. Physiol. Chem. 321,* 90.
Lakowicz, J., & Weber, G. (1973) *Biochemistry 12,* 4171.
Leichtling, B., & Klotz, I. (1966) *Biochemistry 5,* 4026.
Levitt, M., & Greer, J. (1977) *J. Mol. Biol. 114,* 181.
Lumry, R., & Rosenberg, A. (1975) *Colloq. Int. C.N.R.S. 246,* 53.
McCammon, J., Gelin, B., & Karplus, M. (1977) *Nature (London) 267,* 585.
Marinetti, T., Snyder, G., & Sykes, B. (1976) *Biochemistry 15,* 4600.
Masson, A., & Wuthrich, K. (1973) *FEBS Lett. 31,* 114.
Molday, R., Englander, S., & Kallen, R. (1972) *Biochemistry 11,* 150.
Nakanishi, M., & Tsuboi, M. (1974) *J. Mol. Biol. 83,* 379.
Patel, D., & Canuel, L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1398.
Piccinni-Leopardi, C., Fabre, O., & Reisse, J. (1976) *Org. Magn. Reson. 8,* 233.
Richarz, R., & Wuthrich, K. (1977) *FEBS Lett. 79,* 64.
Rosenberg, A., & Chakravarkti, K. (1968) *J. Biol. Chem. 243,* 5193.
Rosenberg, A., & Enberg, J. (1969) *J. Biol. Chem. 244,* 6153.
Saviotti, M. L., & Galley, W. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4154.
Scarpa, J., Mueller, D., & Klotz, I. (1967) *J. Am. Chem. Soc. 89,* 6024.
Scholtan, W., & Lie, S. Y. (1966) *Makromol. Chem. 98,* 204.
Schreier, A., & Baldwin, R. (1976) *J. Mol. Biol. 105,* 409.
Snyder, G., Rowan, R., III, Karplus, S., & Sykes, B. (1975) *Biochemistry 14,* 3765.
Snyder, G., Rowan, R., III, & Sykes, B. (1976) *Biochemistry 15,* 2275.
Stellwagen, E., & Shulman, R. (1973) *J. Mol. Biol. 75,* 683.
Vincent, J., Chicheportiche, R., & Lazdunski, M. (1971) *Eur. J. Biochem. 23,* 401.
Wagner, G., & Wuthrich, K. (1975) *J. Magn. Reson. 20,* 435.
Wagner, G., & Wuthrich, K. (1976) *Protides Biol. Fluids, Proc. Colloq. 23,* 189.
Wagner, G., DeMarco, A., & Wuthrich, K. (1975) *J. Magn. Reson. 20,* 565.
Wagner, G., DeMarco, A., & Wuthrich, L. (1976) *Biophys. Struct. Mech. 2,* 139.
Weast, R., Ed. (1968) *CRC Handbook of Chemistry and Physics* (49th ed) CRC Publishing Co., Cleveland, Ohio.
Weber, G. (1975) *Adv. Protein Chem. 29,* 1.
Welch, W., & Fasman, G. (1974) *Biochemistry 13,* 2455.
Wickett, R., Ide, G., & Rosenberg, A. (1974) *Biochemistry 13,* 3273.
Willumsen, L. (1971) *C.R. Trav. Lab. Carlsberg 38,* 223.
Woodward, C. (1977) *J. Mol. Biol. 111,* 509.
Woodward, C., & Ellis, L. (1975) *Biochemistry 14,* 3419.
Woodward, C., & Rosenberg, A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66,* 1067.
Woodward, C., & Rosenberg, A. (1971) *J. Biol. Chem. 246,* 4105.
Woodward, C., Ellis, L., & Rosenberg, A. (1975) *J. Biol. Chem. 250,* 432.
Wuthrich, K., & Baumann, R. (1976) *Org. Magn. Reson. 8,* 532.
Wuthrich, K., Wagner, G., & Tschesche, H. (1976) *Protides Biol. Fluids, Proc. Colloq. 23,* 201.