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# A Nuclear Magnetic Resonance Study of Bovine Pancreatic Trypsin Inhibitor. Tyrosine Titrations and Backbone NH Groups†

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ABSTRACT: The low-field portion of the 250-MHz $^1H$ nuclear magnetic resonance spectrum of bovine pancreatic trypsin inhibitor has been studied as a function of pH over the range pH 7–13. Of the 12 resonances which are observed downfield from the aromatic region, 11 exchange with the deuterons of the solvent ($D_2O$) with half-times exceeding 4 months. Analysis suggests that these resonances can be assigned to buried NH protons. The 3,5 protons ortho to the hydroxyl group of the four tyrosine residues have been resolved and titrated yielding $pK_A$'s for the four tyrosines of 10.6, 10.8, 11.1, and 11.6. Denaturation of the protein is observed only above pH 13, or, at pH 7, in 8 M urea with excess β-mercaptoethanol.

The basic bovine pancreatic trypsin inhibitor (BPTI)[1] consists of a single polypeptide chain of 58 amino acids with three disulfide bridges. Although it is the smallest globular protein with known X-ray structure (Huber *et al.*, 1970, 1971), it is large enough to contain hydrogen-bonded sections of β sheet and α helix characteristic of other crystalline proteins. The ease of refolding this molecule following either reduction of the bridges of the native inhibitor (Anderer and Hörnle, 1966; Chauvet and Acher, 1966; Avineri-Goldman *et al.*, 1967; Pospisilova *et al.*, 1967) or deblocking of the cysteines in the synthetic inhibitor (Noda *et al.*, 1971) suggests that BPTI may be a suitable system for monitoring the dynamic aspects of protein folding. Known methods for selective disulfide bridge reduction (Liu and Meienhofer, 1968; Meloun *et al.*, 1968a), selective tyrosine modification (Meloun *et al.*, 1968b; Sherman and Kassell, 1968), and controlled sequential proteolytic cleavage (Wilson and Laskowski, 1971) offer a variety of modified derivatives for experimental observation. Moreover, a theoretical understanding of the BPTI structure is simplified by the relatively small number of variable dihedral angles and the conformational constraints imposed by the high density of disulfide cross-links. Two unusual features of the inhibitor are its retention of activity following exposure to high temperatures or pH extremes (Green and Work, 1953a,b; Sherman and Kassell, 1968), and its role in the trypsin–inhibitor complex which has one of the highest known association constants between two macromolecules (Vincent *et al.*, 1971).

Nuclear magnetic resonance studies of protein structures

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[1] Abbreviations used are: BPTI, basic pancreatic trypsin inhibitor; $Me_6Si_2O$, hexamethyldisiloxane.

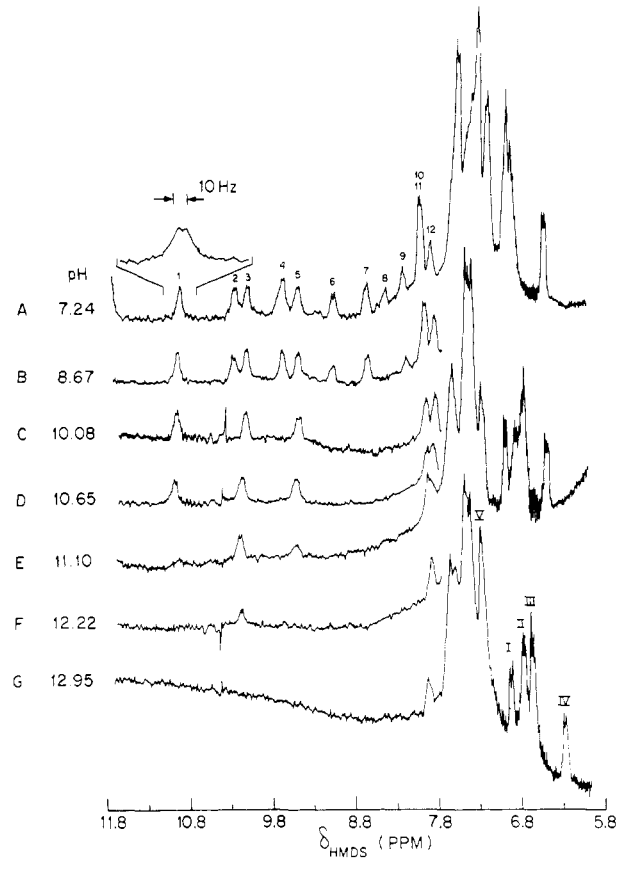


FIGURE 1: Low-field portion ($\delta_{Me_6Si_2O}$ = 11.8–5.8 ppm) of the 250-MHz nmr spectrum of BPTI; [BPTI] $\cong 2.5 \times 10^{-3}$ M in 0.05 M potassium phosphate buffer, 0.1 M KCl in $D_2O$, pH as indicated; 200 scans using correlation spectroscopy method except the expanded region of the pH 7 spectrum which is 300 scans. The 10-Hz scale refers to the expanded plot.

progress in three stages, the preliminary resolution of peaks due to residues having unique magnetic environments, the assignment of those peaks to particular residues, and the use of the assigned peaks as probes of conformations and conformational changes in solution. This report describes the resolution and pH dependence of $^1H$ resonances due to the four tyrosine residues and numerous buried NH's or OH's of BPTI, using 250-MHz nmr correlation spectroscopy (Dadok *et al.*, 1972). With regards to amino acid composition, the inhibitor lacks histidine and tryptophan. These residues were useful in ribonuclease (Bradbury and Scheraga, 1966; Meadows *et al.*, 1968; King and Bradbury, 1971) and lysozyme (Glickson *et al.*, 1969, 1971) studies as a result of the unique downfield chemical shifts of the imidazole and indole hydrogens. Nevertheless, a simpler aromatic region results from the absence of the upfield resonances of these residues which otherwise would overlap with peaks of the four phenylalanine and four tyrosine rings of BPTI. Not only are the upfield doublets of each of the tyrosines resolved in this small stable molecule, thereby indicating the protein is large enough to possess local magnetic environments, but in addition each tyrosine follows a simple titration curve in the region of pH 9–13. This is in contrast to nmr titration curves of the tyrosines of staphylococcal nuclease (Putter *et al.*, 1970; Jardetzky *et al.*, 1971), a large protein with 149 amino acids but no disulfide bridges, in which it is difficult to separate the combined effects of deprotonation and denaturation at high pH. A second interesting observation is the existence of ten resolved peaks, presumed to be buried NH's or OH's, which fail to exchange with $D_2O$ at pH 7 over an interval of 4 months. Although BPTI is too small to possess much of an interior, its rigidity apparently prevents exposure of these hydrogens to solvent molecules.

## Experimental Section

BPTI was obtained from Sigma Chemical Co. and as a gift from Farbenfabriken Bayer AG and was used without further purification. The Sigma BPTI was used only for the high pH denatured sample (see below). L-Ala-L-Tyr was obtained from Sigma Chemical Co., and urea-$d_4$ and $D_2O$ from Stohler Isotope Chemicals.

Seven separate samples (A–G, see Figure 1) were prepared for the pH series. Each consisted of $\simeq 2.5 \times 10^{-3}$ M BPTI in 0.05 M potassium phosphate buffer, 0.1 M KCl in $D_2O$. These samples were adjusted to different pH's using KOD at the time of preparation, and were observed after standing at those pH's for 2 days. For comparison, an eighth sample prepared under similar conditions at pH 7 was observed 4 months after preparation.

Two samples of denatured BPTI are also described in this paper. The pH 7 denatured BPTI sample was prepared as follows. BPTI ($\simeq 3.5 \times 10^{-3}$ M) was added to a $D_2O$ solution 8 M in urea-$d_4$, pH 7.62 (the particular sample used remained in 8 M urea for 2 months). A 100-fold molar excess of $\beta$-mercaptoethanol was then added, and the nmr spectrum was taken after 6 hr. The high pH denatured BPTI sample was prepared as in samples A–G, except that its pH was taken above pH 13 with excess KOD.

$^1H$ nmr spectra at 250 MHz were obtained on a MPC-HF 250-MHz superconducting spectrometer (Dadok *et al.*, 1970). Time averaging at 250 MHz was achieved using correlation spectroscopy. Of the order of 200 rapid scans of the spectrum were digitized and accumulated (1500 Hz sweep width, 3 sec/scan, $1 \times 10^{-3}$ sec filtering) and then correlated with a narrow reference peak ($CHCl_3$) accumulated under identical conditions. The resulting line widths are the sum of the line widths of the sample ($\Delta\nu \simeq$ 8–20 Hz) and reference ($\Delta\nu \simeq$ 0.5 Hz) resonances. Ambient temperature for the 250-MHz spectra was 25°. Nuclear magnetic resonance spectra at 100 MHz were obtained on a Varian HA-100 spectrometer equipped with Fourier transform accessories for time averaging. Ambient temperature for 100-MHz spectra was 32°. Chemical shifts were measured with respect to HDO, and then converted to hexamethyldisiloxane ($Me_6Si_2O$)[1] by measuring the chemical shift of HDO with respect to an external capillary of $Me_6Si_2O$. These chemical shifts have not been corrected for bulk magnetic susceptibility.

pH was measured with a Beckmann pH meter and 39030 combination electrode standardized with Beckmann pH 10.00 buffer. pH's above 11 were not corrected for $K^+$ interference and therefore cannot be considered more accurate than ±0.2 pH unit.

## Results

Figure 1 displays the low-field portion (11.8–5.8 ppm)[2] of the 250-MHz nmr spectra of BPTI samples left standing for 2 days at the indicated pH's. These spectra will be divided

[2] The complete 220 MHz nmr spectrum of BPTI at pH 7.7 and 25° has been reported by Wüthrich (1973).

TABLE I: Chemical Shifts and Coupling Constants of the Low-Field ($\delta_{Me_6Si_2O}$ (ppm) = 11.0–7.8) Resonances in the 250-MHz Nuclear Magnetic Resonance Spectrum of BPTI; pH 7.24; 0.05 M Potassium Phosphate Buffer, 0.1 M KCl in $D_2O$.

| | $\delta_{Me_6Si_2O}$ (ppm) | $J$ (Hz) |
|---|---|---|
| 1. | 10.99 | 4.6 |
| 2. | 10.33 | 7.4 |
| 3. | 10.19 | 7.0 |
| 4. | 9.77 | 4.5 |
| 5. | 9.59 | 6.7 |
| 6. | 9.15 | 7.9 |
| 7. | 8.73 | 5.8 |
| 8. | 8.47[a] | |
| 9. | 8.29 | |
| 10. | 8.04 | |
| 11. | 8.04 | |
| 12. | 7.95 | |

[a] Lifetime in $D_2O$ greater than 2 days but less than 4 months (all of the other resonances have a lifetime in $D_2O$ at pH 7 of greater than 4 months).

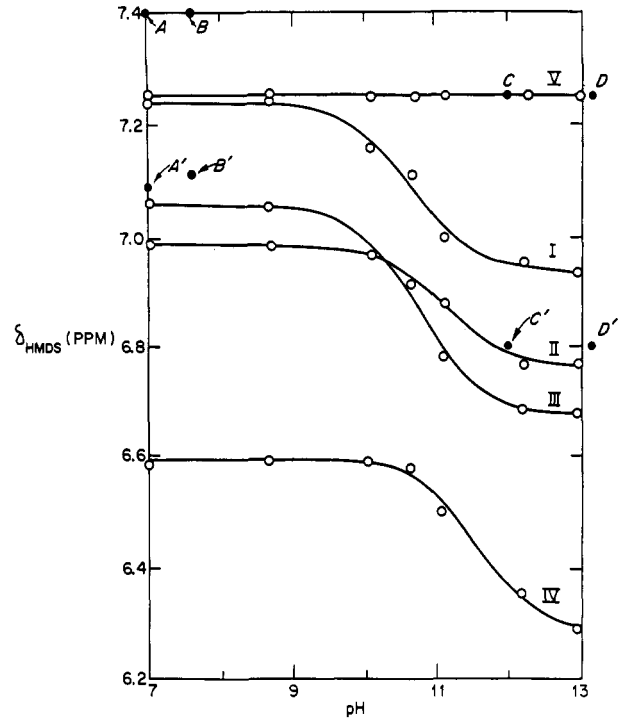


FIGURE 2: Chemical shifts of the five resonances in the region $\delta_{Me_6Si_2O}$ = 7.4–6.2 of the 250-MHz nmr spectrum of BPTI as a function of pH; 0.05 M potassium phosphate buffer, 0.1 M KCl in $D_2O$. Curves I–IV are the calculated nonlinear least-squares fits to single proton titration curves (see Results section for details). Other chemical shifts included are the following where the unprimed points correspond to the 2,6 protons meta to the tyrosine hydroxyl group and the primed points correspond to the 3,5 ortho protons: A and A′, 0.2 M L-Ala-L-Tyr at pH 7 in 0.05 M potassium phosphate buffer, 0.1 M KCl; B and B′, the four tyrosines of "pH 7 denatured" BPTI (100 MHz spectrum, see Experimental Section for details); C and C′, 0.2 M L-Ala-L-Tyr at pH 12 in 0.05 M potassium phosphate buffer, 0.1 M KCl; D and D′, the 4 tyrosines of "high pH denatured" BPTI (pH >13, 100-MHz spectrum, see Experimental Section for details). All chemical shifts for tyrosine resonances (I–IV) correspond to the midpoint of the observed doublets.

into three regions for discussion: 11–7.8 ppm, 7.8–7.4 ppm, and 7.4–6.2 ppm. The isolated doublet (peak IV) appearing between 6.6 and 6.3 ppm in spectra A, E, and G, which later will be assigned to the two nonexchangeable 3,5 protons of one tyrosine ring, serves as a standard peak with area corresponding to two protons.

Sample A, prepared at pH 7.24, contains eleven individual resonances in the region 11–7.8 ppm. All of these resonances have approximately equal areas corresponding to 1 proton, with the exception of the peaks labeled 8 which has an area of less than one proton and "10, 11" which has twice the area of the other peaks. In expanded plots, peaks 1–7 appear as doublets with coupling constants in the range 4–8 Hz. One portion of these plots showing peak 1, the poorest resolved doublet, is included in Figure 1. These resonances are assigned in the discussion section to slowly exchanging NH or OH resonances. The observed chemical shifts and coupling constants are listed in Table I. If spectrum A, pH 7.24 and age 2 days, is compared with a spectrum (not shown) of an equivalent sample, pH 7 and age 4 months, the only change is the absence of peak 8 in the latter. No other peaks decreases in intensity at pH 7.

The individual resonances in the region 11–7.8 ppm disappear, with the exception of peak 12, in the spectra of samples B–G prepared at higher pH's. No additional resonances are observed in this region at the higher pH's. This behavior is shown in Figure 1. For example, peaks 2, 4, and 7 are present at pH 8.67 but absent at pH 10.08; peaks 6, 9, and one-half of the intensity of peak "10, 11" are present at pH 7.24, have lower intensity at pH 8.67, and are absent at pH 10.08; and peak 3 is present at pH 11.10, has lower intensity at pH 12.22, and is absent at pH 12.95. Line broadening and chemical shifts are also observed. Of the five peaks remaining at pH 11.10, peaks 3 and 12 are unshifted and unbroadened with respect to the pH 10.65 sample. However, peak 1 is shifted upfield 10 Hz (at 250 MHz) and is very broad ($\Delta\nu \simeq$ 50 Hz), peak 5 has shifted upfield slightly and is broadened ($\Delta\nu \simeq$ 30 Hz), and the remaining one-half of the 10,11 peak has shifted towards peak 12. These line widths have not been corrected for the doublet character of each resonance ($J \simeq$ 4–8 Hz).

The region of the spectrum of BPTI between 7.4 and 6.2 ppm contains 5 peaks (Figure 1A, E, and G). The four upfield peaks shift as a function of pH and are best resolved at pH 12.95 (peaks I–IV in Figure 1G). The fifth peak does not shift as a function of pH, but increases in area between pH 11 and 12 as if a peak from the region of the spectrum between 7.8 and 7.4 ppm was shifted into this region. The pH dependence of the chemical shift of these five resonances is shown in Figure 2. Each of the four upfield resonances has an area corresponding to two protons, appears as a doublet with an apparent coupling constant of 7–8 Hz, and titrates as a function of pH in the region of pH 10–12. In the Discussion section, these resonances will be assigned to the 3,5 ortho protons of tyrosines-10, -21, -23, and -35.

Each of the titration curves drawn through the observed chemical shifts in Figure 2 is a nonlinear least-squares fit (Arley and Randerbuck, 1950) to a simple one proton titration curve (eq 1)

$$\delta_{obsd} = \delta_{HA}\frac{[H^+]}{[H^+] + K_A} + \delta_A\frac{K_A}{[H^+] + K_A} \quad (1)$$

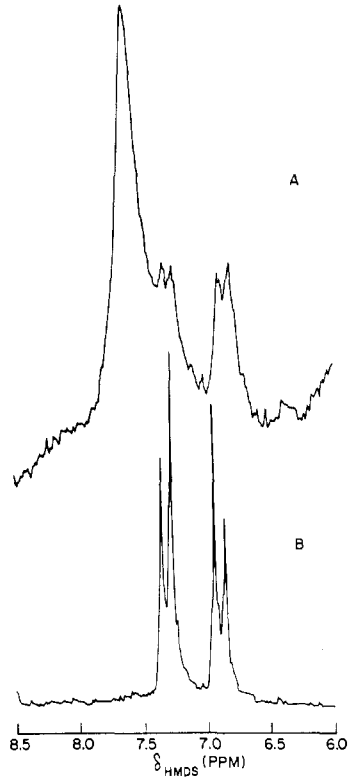


FIGURE 3: (A) Aromatic region of the 100-MHz nmr spectrum of BPTI denatured at high pH (pH >13, see Experimental Section for details), [BPTI] $\cong 2 \times 10^{-3}$ M, 1728 scans using Fourier transform spectroscopy method. (B) Aromatic region of the 100-MHz nmr spectrum of L-Ala-L-Tyr at pH 12; 0.05 M potassium phosphate buffer, 0.1 M KCl; [L-Ala-L-Tyr] = 0.2 M, four scans using Fourier transform spectroscopy method.

TABLE II: Parameters of the Nonlinear Least-Squares Fit of the Chemical Shift of the Four Upfield ($\delta_{Me_6Si_2O}$ (ppm) = 7.4–6.2) Tyrosine Resonances as a Function of pH to Single Proton Titration Curves; 0.05 M Potassium Phosphate Buffer, 0.1 M KCl in $D_2O$.

| | I | II[c] | III[c] | IV |
|---|---|---|---|---|
| $\delta_{HA}$[a] | 7.24 | 7.06 | 6.99 | 6.60 |
| $\delta_{A^-}$[a] | 6.94 | 6.68 | 6.77 | 6.29 |
| $\delta_{HA} - \delta_{A^-}$ | 0.30 | 0.38 | 0.22 | 0.31 |
| $pK_A$ | 10.6 | 10.8 | 11.1 | 11.6 |
| $\sigma$[b] | 4.4 | 4.5 | 2.1 | 3.8 |
| | | II[d] | III[d] | |
| $\delta_{HA}$ | | 7.05 | 7.00 | |
| $\delta_{A^-}$ | | 6.78 | 6.68 | |
| $\delta_{HA} - \delta_{A^-}$ | | 0.27 | 0.32 | |
| $pK_A$ | | 10.7 | 11.0 | |
| $\sigma$ | | 5.3 | 5.0 | |

[a] Chemical shift from $Me_6Si_2O$. [b] Standard deviation of the fit. [c] Resonances II and III chosen as crossing. [d] Resonances II and III chosen as noncrossing.

with $\delta_{HA}$, $\delta_{A^-}$, and $K_A$ as parameters of the fit. Because two of the doublets are superimposed at pH 10.08 and 10.65 giving a peak corresponding to four protons, the chemical shifts representing this peak in Figure 2 were used twice in the fitting, both for peak II and for peak III. Although these two doublets are resolved at lower and higher pH's, ambiguity exists relative to the correspondence between the low pH and high pH peaks. Thus two sets of fits were calculated, with peaks II and III chosen as crossing or non-crossing at the point of superposition.

The results of both sets of fits are presented in Table II. The change in chemical shift between the fully protonated and fully unprotonated species ($\delta_{HA} - \delta_{A^-}$) for each of the tyrosines falls in a narrower range (0.27–0.32 compared with 0.22–0.38) if doublets II and III are chosen as noncrossing. However, the fits are better, as indicated by smaller $\sigma$'s, if the doublets are chosen as crossing. The $pK_A$'s of peaks II and III are independent of this choice. The curves drawn in Figure 2 represent the peaks as crossing.

The aromatic regions of the 100-MHz nmr spectra of high pH (>13) denatured BPTI and of L-Ala-L-Tyr at pH 12 are shown in Figure 3. The trypsin inhibitor spectrum consists of a single peak of 20 protons and two doublets of 8 protons each. These doublets are plotted as points D and D′ in Figure 2. They have the same chemical shift as the tyrosine doublets of L-Ala-L-Tyr at pH 12, plotted as C and C′ in the same figure. Thus, the aromatic region of the nmr spectrum of BPTI denatured at high pH (>13) (see Experimental Section for details) is the superposition of the spectra of the individual aromatic amino acids (one resonance from the four phenylalanines and two doublets from the four tyrosines) at high pH. No peaks occur in the region of 11–7.8 ppm. A small peak in Figure 3 occurs at 6.3 ppm, the same chemical shift as that of peak IV in BPTI at pH 12.95 (Figure 1G).

The pH 7 denatured sample contains BPTI in the presence of urea and $\beta$-mercaptoethanol. During the preparation of this sample, a spectrum was taken just prior to adding the $\beta$-mercaptoethanol. This solution, containing 8 M urea but no reducing agent, had essentially the same spectrum, including downfield peaks, as native BPTI at pH 7. After the addition of reducing agent, the spectrum (not shown) collapses significantly but shows more structure at 250 MHz than the exact superposition of one phenylalanine singlet and two tyrosine doublets at pH 7. No peaks occur in the region of 11–7.8 ppm.

The region of the spectrum between 7.8 and 7.4 ppm contained several peaks which were impossible to unambiguously resolve even at 250 MHz.

## Discussion

The downfield resonances between 11 and 7.8 ppm in the nmr spectrum of BPTI can be assigned to exchangeable NH or OH protons on the basis of their chemical shift, area, behavior as a function of pH, and coupling constants. Studies of small peptides in $Me_2SO$ and $H_2O$ reveal that backbone NH's and amide $NH_2$'s appear downfield from aromatic residues, while tyrosine OH's occur even further downfield than backbone NH's (Glickson *et al.*, 1972). Although serine OH's have been reported upfield of the aromatic region (Kopple, 1971), such resonances could shift into the NH region as the result of hydrogen bonding or aromatic ring current shifts. BPTI contains no histidine or tryptophan so that these resonances cannot be imidazole or indole CH's or NH's. BPTI contains between 99 and 119 exchangeable hydrogens attached to nitrogen and oxygen, the exact number being variable since there are 20 titratable groups. These exchangeable hydrogens

occur in groups of 1–3 hydrogens (for example, peptide NH, glutamine $NH_2$, and lysine $NH_3^+$).

With respect to the area and the behavior of the resonances in the region 11–7.8 ppm as a function of pH, it is important to emphasize that separate samples of BPTI were prepared at different pH's and observed as a function of time. An alternative method of observing pH-induced structural changes is to prepare one sample and titrate it with increments of base. The spectra taken after each increment are then a joint function of the time and pH history of the single sample. The two methods are equivalent for observing the chemical shifts of nonexchangeable tyrosine ring protons as a function of pH, since exposed titratable protons come to equilibrium quickly before spectra are taken. However, for observing the slow chemical exchange of buried NH's in a protein dissolved in $D_2O$ at a given pH, the first method is preferable. The twelve resonances follow a consistent pattern as a function of pH. First, the number of observable peaks decreases monotonically with increasing pH, from 11 at pH 7.24 to 1 at pH 12.95. Secondly, peaks observable in samples B–G occur in essentially the same positions occupied by the 11 peaks of sample A. For these reasons, the decrease in intensity of a peak in samples B–G is attributed to an increased rate of chemical exchange with $D_2O$. Similarly, the loss of intensity is attributed to complete exchange, rather than a drastic change in chemical shift or line width. This conclusion will be supported in a later discussion of the upfield region of the spectrum, where the tyrosine titrations indicate no abrupt changes in BPTI structure between pH 7 and 13.

The pH 7 samples were observed after 2 days and after 4 months. After 2 days, peak 8 had an area corresponding to less than one hydrogen indicating a half-life on the order of days at pH 7. After 4 months, it was completely exchanged as expected. All other regions of the spectrum are unchanged, indicating no structural changes or denaturation on sitting for several months. In fact, BPTI may be kept for 18 months at room temperature in 0.14 M NaCl without loss of activity (Sach *et al.*, 1965). Since all other exchangeable peaks have equal intensities at both times, the corresponding $\tau_{1/2}$'s must be $\gg$4 months, with initial intensities equal to intensities observed after two days. Thus the groups represented in Figure 1A by peaks 9 and 1–7 each contain only one exchangeable hydrogen. Peak "10,11" corresponds to a group containing two such hydrogens, or to the superposition of two groups with one hydrogen each. The initial number of hydrogens in group 8 is unknown. No loss of intensity was observed for peak 12 before denaturation.

Some restrictions on assignments can be made, based on the doublet nature of peaks 1–7 (see Table I). Among the groups containing one exchangeable hydrogen, 50 could occur as doublets: the 3 threonine OH's and the 47 nonglycine backbone N(H)'s. In addition, it is possible for the protons of an immobilized $NH_2$ group to have nonequivalent chemical shifts and to couple to each other as doublets (Brodie and Poe, 1971).[3] The apparent coupling constants, 4–8 Hz, are in the same range observed for $J_{HN-C\alpha H}$ of backbone NH's in small peptides in $Me_2SO$ and $H_2O$ (Kopple, 1971). $J_{HO-C\beta H}$ for threonine is expected to be in the same range (Barfield and Karplus, 1969).

Observation of a half-time for exchange of $\gg$4 months at pH 7 is indicative of hydrogens shielded from contact with solvent molecules. The monotonic increase of exchange rate with pH therefore indicates that the buried hydrogens become increasingly exposed at higher pH's. These conclusions are based on observations of exchange rates in amino acids, small peptides, and other proteins. In isolated amino acids, hydrogens attached to O and S exchange too rapidly to be observed in $D_2O$ solutions (Glickson *et al.*, 1971). Hydrogens attached to nitrogen can have half-lives as long as several hours, especially at low pH's where base-catalyzed exchange is minimized (Brewster and Bovey, 1971). Buried exchangeable hydrogens are observed in fresh $D_2O$ solutions of such proteins as lysozyme (McDonald *et al.*, 1971), ribonuclease (Roberts *et al.*, 1969), cytochrome *c* (McDonald and Phillips, 1970), and staphylococcal nuclease (Putter *et al.*, 1970). In lysozyme, some exchangeable protons remain in the spectra after several days of standing in $D_2O$ below 45°. Some cytochrome *c* protons exchange more slowly than those of lysozyme. Insufficient data currently exist to permit comparisons of slowly exchanging buried hydrogens with respect to such factors as protein size, number of disulfide bridges, or number of buried water molecules.

The X-ray crystal structure of BPTI (Huber *et al.*, 1970, 1971) offers additional information with respect to which exchangeable hydrogens are buried in the molecule. A space-filling model of BPTI was constructed using the dihedral angle technique (Yankeelov and Coggins, 1972) and coordinates generously supplied by R. Huber. Inspection of the model shows that the exchangeable hydrogens on groups which are likely to be charged at pH 7 are completely exposed on the exterior of BPTI. These groups include the backbone amino- and carboxyl-terminal ends and the carboxyl, amino, and guanidino ends of all aspartate, glutamate, lysine, and arginine side chains. Among the remaining exchangeable side-chain groups, several seem to be partially buried within the molecule and hence may contribute to peaks 1–12 in Figure 1. These groups include the OH of threonine-54, the $\epsilon$ NH's of arginines-1 and -20, and the $\gamma$ $NH_2$'s of asparagines-43 and -44. Several backbone NH's are buried, especially that of residue 22 which is not visible from the exterior of the space-filling model. These observations therefore suggest that with the exception of one possibly buried hydroxyl group, all exchangeable downfield resonances are NH's.

The line broadening in peaks 1 and 5 at pH 11.10 could be the result of either a static or a dynamic change in structure at that pH. For example, a change in conformation going from pH 10.65 to 11.10 might bring a hydrogen atom into a position closer to hydrogen 1, resulting in broadening by increased dipole–dipole relaxation. However, such a mechanism could account for only a small portion of the 30-Hz broadening observed.[4,5] Alternatively, if an equilibrium exists at pH 11.0 between two conformations of the protein, the broadening may be due to the exchange of hydrogens 1 and 5 between more than one magnetic environment. The observed line broadening is not due to the onset of exchange with

[3] The authors are indebted to a referee for pointing out this possibility.

[4] Assuming for calculation purposes that two protons 2.0 Å apart are undergoing isotropic rotational motion with a $\tau_c$ appropriate for BPTI of $1 \times 10^{-9}$ sec (Bradbury *et al.*, 1971), then

$$\Delta\nu(\text{dipole–dipole}) = \frac{1}{\pi T_2} = \frac{3\hbar^2\gamma^4}{40\pi r^6}\left[6\tau_c + \frac{10\tau_c}{1+(\omega_0\tau_c)^2} + \frac{4\tau_c}{1+4(\omega_0\tau_c)^2}\right] \cong 2.0 \text{ Hz}$$

[5] This indicates in turn that dipole–dipole interactions are relatively unimportant with respect to scalar interactions with $^{14}N$ in determining the observed initial line widths of all of the NH peaks.

the solvent because the rate is so fast that all of the H's would have exchanged for D's in much less than 2 days.[6] Also, broadening due to dimerization can be ruled out since slower tumbling of the dimer would broaden all peaks simultaneously. Furthermore, BPTI is known to shift from dimer to monomer on increasing pH above 7 (Kraut *et al.*, 1960; Scholtan and Lie, 1966).

A comparison of the pH 10.65 and 11.10 spectra shows that changes in line width, chemical shift, and intensity occur in peaks 1, 5, and 11 without affecting peaks 3 and 12. This is indicative of local conformational changes, rather than concerted changes occurring throughout the entire molecule and affecting all peaks simultaneously. These local changes are probably small in nature and do not reflect major discrete states of unfolding since the tyrosine titrations, to be discussed below, indicate no significant denaturation of the protein between pH 7 and 13.

Most of the local changes occur in two pH regions. For example, peaks 2, 4, and 7 undergo major changes in exposure to solvent in the region between pH 8.7 and 10.1. Changes in peaks 1, 5, and 11 between pH 10.7 and 11.1 have already been mentioned. The first region corresponds to the expected titration region of the terminal backbone $NH_2$ of the first amino acid in the chain. CD–ORD studies on BPTI show a conformational change occurring with a pK of 9.4 assigned to the terminal $NH_2$ (Vincent *et al.*, 1971). The second region corresponds to the region of tyrosine and lysine titrations. The observed chemical shifts are consistent, for example, with the titration and concomitant movement of tyrosine residues. Other BPTI reports based on studies of aromatic uv absorption (Scholtan and Rosenkranz, 1966; Sherman and Kassel, 1968) or tyrosine fluorescence (Cowgill, 1967) suggest conformational changes associated with tyrosine titrations with p$K$'s between 10 and 11.

The four upfield doublets I–IV (Figure 1) in the region 7.4–6.2 ppm correspond to BPTI's tyrosines. This assignment is based on their nonexchangeability, p$K$'s of 10–12, area of two protons, apparent coupling constants of 7–8 Hz, and chemical shifts in the aromatic region. From the aromatic region of the spectrum of L-Ala-L-Tyr (Figure 3B), it can be seen that the tyrosine ring's spectrum consists of two doublets. The downfield doublet, corresponding to the 2,6 meta protons (Martin and Morlino, 1965), shifts upfield 0.15 ppm (Figure 2) on going from the fully protonated to fully unprotonated OH ($\delta_{HA} - \delta_{A^-}$). This is half the value (0.30 ppm) for the upfield shift of the 3,5 ortho protons. The 2,6 meta proton doublet of tyrosine has approximately the same chemical shift as the aromatic proton resonance of phenylalanine, even at high pH (see, for example, Figure 3A). This is closely analogous to the spectrum of histidine (King and Bradbury, 1971), in which the C-2 proton is well resolved from phenylalanine but the C-4 proton is not, and in which one of the ring protons shifts half as much (0.5 ppm compared with 1.0 ppm) as the other during titration of the NH. However, the separation in chemical shifts between the downfield and upfield peaks is considerably less in tyrosine (0.3–0.45 ppm) than in histidine (0.65–1.05 ppm).

In ribonuclease, all eight peaks of the four histidines have been resolved by difference spectroscopy (King and Bradbury, 1971) and assigned by selective chemical modification (Meadows *et al.*, 1968). Only four of the eight doublets of the four tyrosines in BPTI have been resolved. Unambiguous assignment of the BPTI peaks to 2,6 or 3,5 doublets and to particular residues requires further work. However, several features suggest that the four observed peaks are the upfield doublets of each of the four rings. In the first place, peaks I–IV have approximately the same chemical shifts at any pH as the 3,5 doublet of L-Ala-L-Tyr, although the downfield and upfield doublets of L-Ala-L-Tyr are close enough that it would be possible for local magnetic environments to shift the downfield doublet of one residue upfield from 7.2 ppm and the upfield doublet of another residue downfield from 7.2 ppm. Secondly, ($\delta_{HA} - \delta_{A^-}$) for the peaks are the same as that for the upfield L-Ala-L-Tyr doublet except for peak III crossing where the difference is 0.22 ppm, midway between the L-Ala-L-Tyr 2,6 (0.15) and 3,5 (0.30) values. The choice of crossing instead of noncrossing titration curves discussed earlier is based solely on better fitting to single proton titration curves. Thirdly, if two of the four BPTI peaks were associated with the same ring, they should have the same p$K_A$'s, as is observed in the ribonuclease work. Only peaks II and III of BPTI have almost the same p$K$'s. It is unlikely that pairs I–III, I–IV, II–IV, or III–IV belong to the same ring.

The calculation of tyrosine p$K_A$'s is based upon single proton titration curves (eq 1). This assumes that no other titratable group influences the chemical shift of a given tyrosine in the region in which it titrates. This assumption is not necessarily valid for a given tyrosine which has a p$K$ of 10–11 with lysines, arginines, other tyrosines, and the terminal $NH_2$ of the first amino acid also titrating between pH 9.5 and 12.5. This is reflected in the complicated tyrosine titration curves of staphylococcal nuclease (Putter *et al.*, 1970; Jardetzky *et al.*, 1971), where denaturation occurs concurrently with deprotonation. It appears however that each of the BPTI tyrosines does follow a single proton titration curve, and that BPTI does not denature significantly after 2 days at pH's up to 13. Its physiological activity has been reported to remain constant for 24 hr at room temperature up to pH 12.6, but begins to decrease at pH 12.8 (Sherman and Kassell, 1968). The different behavior of staphylococcal nuclease and BPTI may be related to the complete lack of disulfide bridges in the nuclease and the relatively high density of bridges in the inhibitor.

BPTI is not denatured in 8 M urea at pH 7 if its disulfide bridges are intact, but it is denatured upon addition of excess reducing agent, when the bridges are presumed to be broken. The nmr spectrum of BPTI in 8 M urea is essentially the same as that of native BPTI, including the observation of individual NH resonances and unique local magnetic environments for tyrosines. CD–ORD spectra of BPTI indicate no significant conformational changes in 0–7 M guanidine-HCl or 0–10 M urea (Vincent *et al.*, 1971). However, on addition of excess $\beta$-mercaptoethanol, all tyrosines have approximately the same chemical shift and the NH's are completely exchanged. Similar results occur with lysozyme (Cohen and Jardetzky, 1968), a molecule of twice the size of BPTI, and containing one additional bridge. Essentially complete denaturation of BPTI also occurs after two days of exposure to high salt and pH >13. This is indicated by the lack of individual magnetic environments resulting in the superposition of phenylalanine peaks into one singlet and of tyrosine peaks into two doublets (Figure 3). Since desulfuration of bridges can occur at high pH (Vincent *et al.*, 1971) and quantitative assays for disulfide bridging were not performed, the state of the bridges is uncertain in the high pH denatured sample.

[6] Assuming for calculation purposes that the chemical shift of 10 Hz represents a weighted average of two approximately equally populated sites and that the line broadening represents the intermediate exchange region ($(\tau\Delta)^2 \simeq 1$), then $\tau \simeq 0.01$ sec.

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