Complete Tyrosine Assignments in the High Field ^1H Nuclear Magnetic Resonance Spectrum of the Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: The low-field portions of the 250-MHz ^1H nuclear magnetic resonance (NMR) spectra of native and chemically modified bovine basic pancreatic trypsin inhibitor (BPTI) have been studied as a function of pH over the range pH 5–13. Resonances associated with the 16 protons of the aromatic rings of the four BPTI tyrosines have been located and assigned to specific tyrosyl residues. Titrations of pH yielded pK's for tyrosines-10, -21, -23, and -35 of 10.4, 11.0, 11.7, and 11.1, respectively. The resonances associated with the nitrotyrosine-10 protons of mononitrated BPTI and the nitrotyrosine-10 and -21 protons of dinitrated BPTI have been similarly located, assigned and titrated yielding pK's for nitrotyrosine-10 and -21 of 6.6 and 6.4, respectively. The high-field NMR spectrum indicates that the aromatic ring of tyrosine-35 rotates less than 160 times per second at 25° for pH's in the range 5–9.

The basic bovine pancreatic trypsin inhibitor (BPTI)[1] consists of a single polypeptide chain of 58 amino acids with three disulfide bridges. The complexes formed between the inhibitor and either trypsin or chymotrypsin are of interest in the study of protein–protein interactions. The kinetics and thermodynamics of formation of these complexes have been studied (Vincent and Lazdunski, 1972, 1973; Quast et al., 1974), and the crystal structures of both the complex (Huber et al., 1974) and of its constituents (Birktoft and Blow, 1972; Deisenhofer and Steigemann, 1974; Stroud et al., 1974) are known. BPTI itself serves as an ideal system for investigations of the problem of protein folding. Many potential intermediates in the folding–unfolding process can be obtained either by selective cleavage of peptide bonds (Wilson and Laskowski, 1971; Dyckes et al., 1974; Jering and Tschesche, 1974) and of disulfide bridges (Liu and Meienhofer, 1968; Meloun et al., 1968a) in the folded native inhibitor or by selective formation of disulfide bridges in the unfolded reduced inhibitor (Creighton, 1974a,b).

Nuclear magnetic resonance (NMR) studies of protein structures 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. In BPTI, at least a dozen resonances occurring downfield of 7.9 ppm have been assigned to buried backbone NH's on the basis of their exchangeability (Karplus et al., 1973; Masson and Wuthrich, 1973). These have been further assigned to general regions of the known crystal structure on the basis of studies of rates of exchange with D_2O solvent in derivatives obtained by cleavage of peptide bonds and disulfide bridges (Wagner and Wüthrich, 1974). Several resonances upfield of 7.0 ppm have been assigned to tyrosine protons on the basis of their titratability with tyrosine-like pK's (Karplus et al., 1973). This manuscript describes the complete resolution of resonances associated with all 16 tyrosine protons, their complete assignment to specific tyrosine rings, and their partial assignment to positions within those rings. Examples of aromatic proton assignments in proteins other than BPTI include studies of the histidine resonances of ribonuclease (Meadows et al., 1968; King and Bradbury, 1971), the tryptophan resonances of lysozyme (Glickson et al., 1969, 1971), and the tyrosine resonances of staphylococcal nuclease (Jardetzky et al., 1971).

Several features of the inhibitor's structure and chemistry facilitated the assignment of the tyrosine resonances to specific residues. First of all, the inhibitor has a low molecular weight of 6500, resulting in relatively narrow ^1H NMR resonances. Secondly, BPTI has a simple aromatic composition consisting of four phenylalanines and four tyrosines but no histidines or tryptophans. Thus the aromatic portion of the spectrum consists of resonances associated with only 16 tyrosine and 20 phenylalanine protons, the two types being distinguishable on the basis of tyrosine's titratability. Thirdly, as summarized in Table I, each of the four tyrosines has a unique susceptibility to chemical modification (Meloun et al., 1968b; Sherman and Kassell, 1968), thereby permitting a complete and unique assignment of tyrosine resonances to specific residues independently of inferences drawn from knowledge of the crystal structure. Finally, BPTI is stable and soluble over a broad pH range from pH 2.1 (Vincent et al., 1971) to pH 12.6 (Sherman and Kassell, 1968; Karplus et al., 1973) and at high or low pH does not form intermolecular aggregates with concomitant line

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[1] Abbreviations used are: BPTI, basic pancreatic trypsin inhibitor; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid).

Table I: BPTI Tyrosine Chemical Modification.

Derivative[a]	Tyrosine[b]			
N	10	21	35	23
M	NO_2-10	21	35	23
D	NO_2-10	NO_2-21	35	23
I	I-10	I-21	I_2-35	23

[a] N, native; M, mononitrated; D, dinitrated; I, iodinated. [b] NO_2, 3-nitro; I, 3-iodo; I_2, 3,5-diiodo.

broadening (Scholtan and Lie, 1966).

The BPTI tyrosine assignments are made by using a variety of techniques which give a single consistent solution to the assignment problem. These techniques include the comparison of derivatives obtained by chemical modifications of tyrosine, observation of decoupling and negative nuclear Overhauser enhancements in double resonance experiments, analyses of the intensity patterns of resolved doublets, and quantitative determinations of p*K*'s, Hill coefficients, and relative changes in chemical shift derived from pH titrations of BPTI and its derivatives.

The results obtained by these techniques reveal that BPTI's four tyrosines exhibit significant heterogeneity of behavior. A unique feature of the NMR results is the demonstration that tyrosine-35 is immobile in the protein on the NMR time scale at 25° over the pH range 5–9 while the other three tyrosines have a greater degree of internal rotation.

Experimental Section

BPTI was obtained as gifts from Farbenfabriken Bayer AG (Elberfeld, Germany) and Laboratoire Choay (Paris, France). Low molecular weight impurities were removed by ultrafiltration at room temperature in an Amicon dialysis cell using a UM2 membrane and 30 mM ammonium bicarbonate buffer of pH $\simeq$7.9. The protein solution was frozen at −30° and lyophilized to yield purified BPTI. *N*-Acetyl-L-tyrosineamide and *N*-acetyl-3-nitro-L-tyrosine ethyl ester were obtained from Sigma Chemical Company and used without further purification. Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) was obtained from Calbiochem. D_2O, DCl, and acetic-d_4 acid were obtained from Stohler Isotope Chemicals.

Mononitrated and dinitrated BPTI were prepared using a procedure based on modifications of an earlier study (Meloun et al., 1968b). Dilute BPTI solutions (10^{-4} *M*) were nitrated with an ethanolic solution of tetranitromethane (Riordan and Vallee, 1972). Following gel filtration with Sephadex G-25 fine to remove excess reagent and low molecular weight by-products, the protein was concentrated by ultrafiltration and purified by gel filtration on Sephadex G-50 fine to remove polymerized BPTI byproducts. Monomeric BPTI species then were separated into native, mononitrated, and dinitrated derivatives by ion-exchange chromatography on CM-Sephadex 25 (Meloun et al., 1968b), followed by the ultrafiltration desalting method described above. Amino acid analyses confirmed identification of the mono- and dinitrated derivatives. Exhaustively iodinated BPTI was prepared following procedures of an earlier study (Sherman and Kassell, 1968) modified by the periodic addition of iodine in aliquots (Covelli and Wolff, 1966) and ultrafiltration desalting. The assignments in this manuscript depend on the determination of the modified tyrosine residues reported in the earlier studies of BPTI nitration and iodination.

Table II: BPTI Samples—pH's and Derivatives (N, native; M, mononitrated; D, dinitrated; I, iodinated).

N	M	D	I
4.80	4.68	4.83	
	4.98		
	5.50		
	5.81		
	6.13	6.08	
	6.43		
	6.69		
7.0	7.02	6.97	7.0
7.24			
	7.39		
		7.77	
		8.43	
8.50	8.50	8.50	8.50
8.67			
		9.32	
10.08			
10.65			
		10.81	
11.10			
12.22			
12.50	12.50	12.50	12.50
12.95			

Native BPTI was preexchanged by exposure to guanidine-DCl in D_2O at high temperature. This procedure is based on earlier NMR studies of the exchangeable NH's of BPTI (Masson and Wüthrich, 1973). The easily exchangeable protons of Pipes, guanidine-HCl, and BPTI were first removed by repeated lyophilization from D_2O. A solution of 10 m*M* BPTI was then prepared in 6 *M* guanidine-DCl and 40 m*M* Pipes in D_2O at pH 7.0. After exposure to 82° for 30 min, the sample was slowly cooled to room temperature, diluted tenfold with 10 m*M* pyridine in D_2O, adjusted to pH 7.0 with acetic-d_4 acid, and desalted by ultrafiltration using the same pyridine buffer. Native, nitrated, and iodinated BPTI were also preexchanged by exposure to high pH in D_2O, a procedure based on NMR studies of the basic titration of BPTI (Karplus et al., 1973). In this case, the easily exchangeable protons of K_2HPO_4 and BPTI were first removed by repeated lyophilization from D_2O. A solution of 3 m*M* BPTI was then prepared in 15 m*M* K_2DPO_4 in D_2O. After the sample was exposed to pH 12.5 at room temperature for 100 min, the sample was lowered to pH 7 and desalted as in the guanidine-high temperature procedure. The high-pH method is easier to perform than the guanidine-high temperature method and therefore was the procedure of choice following the demonstration that both procedures are equivalent (see Results).

Protein and peptide samples were prepared in D_2O buffers 0.1 *M* in KCl and 0.5 m*M* in DSS; 15 m*M* acetate was used to buffer between pH 4 and 6; 15 m*M* tris(hydroxymethyl)aminomethane was used between pH 6 and 10 and 15 m*M* phosphate in the pH interval 10–13. Occasionally combination buffers were prepared with two or all three of these compounds to permit titration of a single sample over several pH intervals. All BPTI samples are listed in Table II and are $\sim 2.5 \times 10^{-3}$ *M* in preexchanged protein. Titrations of amino acid standards were performed on samples 10 m*M* in peptide. *N*-Acetyl-L-tyrosineamide was studied in the pH interval 5–13 and *N*-acetyl-3-nitro-L-tyrosine ethyl ester between pH 5 and pH 9.5. pH's were adjusted

with DCl and KOD. pH was measured with a Radiometer PHM26 meter and GKS73041 HA-1 electrode calibrated at pH 13 with standardized 0.1 *M* KOH. Additional standardization was against Beckman buffers of pH 4.00, 7.00, and 10.00. pH's above 11 were not corrected for K^+ interference and therefore cannot be considered more accurate than ±0.2 pH unit.

^{1}H NMR spectra at 100 MHz were obtained on a Varian XL-100-15 NMR spectrometer operating in the Fourier transform mode and modified for homonuclear spin decoupling. The spectrometer was locked on D_2O, and the operating temperature was 28°. Spectra at 250 MHz were obtained on the MPC-HF 250-MHz superconducting spectrometer (Dadok et al., 1970). Time averaging at 250 MHz was achieved using correlation spectroscopy (Dadok and Sprecher, 1974) with the spectrometer locked on the residual HDO peak of the sample. On the order of 200–1000 rapid scans of the spectrum were digitized and accumulated (≈1500-Hz sweep width, 1.6 sec/scan) and then correlated with a calculated reference line with a line width of 0.5 Hz. Modifications of the instrument for decoupling are described elsewhere (Dadok et al., 1972). Ambient temperature for 250-MHz spectra was 25°. Spectra at 270 MHz were obtained on a Bruker HX-270 NMR spectrometer operating in the CW mode and locked on the residual HDO resonance of the sample. Conventional time averaging (200 scans) was performed under relatively slow sweep conditions (sweep width 900 Hz, sweep time 200 sec, filter time constant 0.05 sec, 8K data points). The rf field strength was adjusted to be below saturation for the narrow resonances of standard dipeptide samples. The ambient temperature was 27°. All chemical shifts are expressed relative to a DSS internal standard.

Results

To facilitate interpretation of the spectra of native and nitrated BPTI, *N*-acetyl-L-tyrosineamide and *N*-acetyl-3-nitro-L-tyrosine ethyl ester were studied as models of the unmodified and nitrated tyrosine ring. The amide was studied over the range pH 5–13 and the ester between pH 5 and pH 9.5. The ester hydrolyzes above pH 9.5, as observed by the appearance of ethanol resonances in the spectrum. The 100-MHz titration results were similar to those observed in unblocked peptides (Cohen et al., 1971). In Figure 1, the 250-MHz pH 4.8 spectra of the model compounds illustrate typical features of the spectra of tyrosine and nitrotyrosine ring protons. The aromatic portion of tyrosine's spectrum consists of two doublets of area two. The downfield doublet corresponds to the two equivalent 2,6 protons meta to the hydroxyl group and the upfield doublet to the equivalent 3,5 ortho protons. The 8-Hz coupling constant arises from the strong coupling between adjacent protons. Fine structure due to weaker coupling between nonadjacent protons is not visible with the resolution obtainable for tyrosines in a molecule the size of BPTI. Following nitration at position H-3, the 2,6 meta protons now are inequivalent yielding separate resonances of area one with chemical shifts in the order shown. The H-2 resonance will now appear as a singlet at the line widths obtainable for BPTI. These spectral features occur at all pH's between pH 5 and 13. The ring position assignments are based on studies of derivatives selectively deuterated at ortho positions (Martin and Morlino, 1965; Cohen et al., 1971).

The titrations of tyrosine and nitrotyrosine are exemplified by unmodified and nitrated tyrosine-10 in BPTI presented in Figure 8. For tyrosine, both doublets maintain a constant chemical shift below pH 8.5 and then titrate upfield on raising the pH above 8.5. These doublets give an identical p*K* but shift by different amounts in the titration to high pH, with the magnitude of the change greater for the resonance associated with protons ortho to the titrating hydroxyl group. For nitrotyrosine, the three resonances titrate upfield on raising the pH from 4.8 to 8.5, but maintain a constant chemical shift above pH 8.5. The magnitude of the changes in chemical shift upon titration are in the order H-5 > H-6 > H-2. If the tyrosine and nitrotyrosine spectra were superimposed, resonances would overlap in the pH range around 8.5, as seen for ring 10 in BPTI. However, this overlap does not persist throughout the range pH 5–13, since the nitrotyrosine resonances can be shifted downfield below pH 8.5 and the tyrosine resonances can be shifted upfield above pH 8.5.

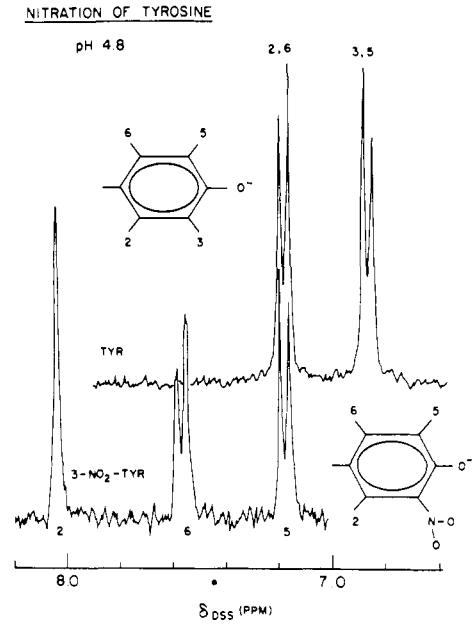

FIGURE 1: Aromatic region (δ_{DSS} 8.2–6.6 ppm) of the 250-MHz NMR spectrum of amino acid standards; Tyr, *N*-acetyl-L-tyrosineamide, 3-NO_2-Tyr, *N*-acetyl-3-nitro-L-tyrosine ethyl ester; [peptide], ≃10 m*M* in 15 m*M* acetate buffer, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O (pH 4.80); ≃25 scans using correlation spectroscopy method.

The observed chemical shifts in the titrations of tyrosine and nitrotyrosine were fit using nonlinear least-squares methods to a one proton titration curve (eq 1) (Markley, 1973):

$$(\delta_{obsd} - \delta_{HA})/(\delta_{A^-} - \delta_{HA}) = K_a^n/(K_a^n + [H^+]^n) \quad (1)$$

with δ_{HA}, δ_{A^-}, K_a, and n as parameters of the fit. The values obtained for δ_{HA}, δ_{A^-}, and K_a did not change within experimental error for either the model compounds or the protein titrations when n was fixed at a value of 1.0, the value corresponding to simple, noncooperative ionizations. The results are summarized in the bottom of Table III. Parameters for resonances associated with ortho and meta protons on the same ring are compared in Table IV. The model compound results demonstrate quantitatively that protons on the same ring exhibit identical p*K*'s and Hill coefficients since they are influenced simultaneously by deprotonation at the ring's hydroxyl group.

The exchangeable protons of native BPTI were preexchanged by either exposure of the protein to high pH in D_2O or to guanidine-DCl and elevated temperatures in D_2O

Table III: Parameters of the Nonlinear Least-Squares Fit of the Chemical Shift of BPTI and Peptide Tyrosine and Nitrotyrosine Resonances as a Function of pH to Single Proton Titration Curves.

Resonance Ring	Proton Position	Comment[d]	Parameters				
			δ_{HA}[a]	δ_{A^-}[a]	pK[b]	n	σ
10	2,6		7.39	7.20	10.6	0.6 ± 0.2	
	3,5		7.15	6.71	10.3	0.7 ± 0.2	
21	2,6	C	6.76	6.55	11.1	1.1 ± 0.2	0.94
	3,5	C	6.83	6.45	10.9	0.9 ± 0.1	1.29
23	2,6		7.23	7.04	11.7	1.4 ± 0.3	
	3,5		6.38	6.07	11.7	0.8 ± 0.2	
35	Downfield		7.83	7.41	11.1	1.9 ± 0.5	
NO_2-10	2	M/D	8.38	8.05	6.6	1.0 ± 0.1	
		M	8.38	8.03	6.6	0.9 ± 0.1	
		D	8.37	8.05	6.7	1.0 ± 0.1	
	6		7.66	7.30	6.6	1.0 ± 0.1	
	5		7.58	7.06	6.7	1.0 ± 0.0	
NO_2-21	2		7.64	7.46	6.3	1.3 ± 0.4	
	6	C	7.10	6.85	6.5	1.4 ± 0.2	0.89
	5	C	7.19	6.70	6.4	1.2 ± 0.1	0.52
21	Downfield	N	6.84	6.54	10.8	0.7 ± 0.1	1.67
	Upfield	N	6.76	6.46	11.0	1.6 ± 0.2	1.37
NO_2-21	Downfield	N	7.19	6.84	6.2	1.0 ± 0.2	1.09
	Upfield	N	7.10	6.70	6.6	1.5 ± 0.1	0.81
Tyr[c]	2,6		7.16	6.99	10.0	1.2 ± 0.1	
	3,5		6.84	6.55	10.0	1.2 ± 0.1	
NO_2-Tyr[c]	2		7.99	7.71	7.1	1.0 ± 0.0	
	6		7.54	7.20	7.1	1.0 ± 0.0	
	5		7.14	6.73	7.1	1.0 ± 0.0	

[a] Chemical shift (±0.02 ppm) from DSS. [b] ±0.2 pK unit. [c] Tyr, *N*-acetyl-L-tyrosineamide; NO_2-Tyr, *N*-acetyl-3-nitro-L-tyrosine ethyl ester. [d] C, resonances chosen as crossing; N, resonances chosen as noncrossing; M, mononitrated BPTI only; D, dinitrated BPTI only; M/D, combined data from mono- and dinitrated BPTI.

Table IV: Comparison of Titration Parameters for Resonances of Ortho and Meta Protons on the Same Ring.

Ring	Comment[d]	Ortho/Meta Comparison[b]			
		ΔpK[a]	Δn[a]	$\Delta(\delta_{HA} - \delta_{A^-})$[a]	Av σ
10		0.3	0.1	0.25	
21	C	0.2	0.2	0.17	1.12
	N	0.2	0.9	0.00	1.52
23		0.0	0.6	0.12	
NO_2-10		0.1	0.0	0.16	
NO_2-21	C	0.1	0.2	0.24	0.71
	N	0.4	0.5	0.05	0.95
Tyr[c]		0.0	0.0	0.12	
NO_2-Tyr[c]		0.0	0.0	0.07	

[a] Using absolute values of the difference. [b] Unmodified rings, comparison of 2,6 and 3,5 resonances; nitrated rings, comparison of H-6 and H-5 resonances. [c] Tyr, *N*-acetyl-L-tyrosineamide; NO_2-Tyr, *N*-acetyl-3-nitro-L-tyrosine ethyl ester. [d] C, resonances chosen as crossing; N, resonances chosen as noncrossing.

(see Experimental Section for details). At pH 8.5 the spectra of the resulting protein obtained by the two methods were identical and lacked resonances occurring downfield of 7.9 ppm which previously were assigned to exchangeable backbone NH's. The high-pH exchanged inhibitor was examined at pH 12.22 using 270-MHz NMR under conditions which produce accurate peak areas. The resultant spectrum appeared much like the one presented at the top of Figure 3. Using the resolved upfield doublet at 6.15 ppm as a standard tyrosine doublet of area two, the area of the aromatic region corresponded to 36 aromatic protons (with an error of approximately ±2 protons) with no indication of the presence of additional exchangeable protons. Specifically, in native BPTI, the resonance at 7.74 ppm corresponds to one proton, the doublets at 6.71 and 6.15 ppm correspond to two protons each, and the multiplet centered at 6.51 ppm corresponds to seven protons. The region between 7.5 and 6.9 ppm, which will be referred to as the phenylalanine region in the remaining discussion, corresponds to the remaining 24 aromatic protons.

As seen in Table II, BPTI derivatives containing different tyrosine modifications were prepared at five pH's, namely 4.8, 6.1, 7.0, 8.5, and 12.5 for direct comparison. Inspection of Table I indicates that pairwise comparisons of spectra of native and mononitrated BPTI will reveal changes resulting from nitration of tyrosine-10, whereas pairwise comparisons of spectra of mononitrated and dinitrated BPTI will reveal changes resulting from nitration of tyrosine-21. The results of one such comparison are illustrated in Figure 2, consisting of the superposition of spectra of native and mononitrated BPTI at pH 4.8. Prior to superposition, the spectrum of native BPTI was scaled to give the upfield doublet of area two at 6.37 ppm the same intensity observed in the mononitrated spectrum. The spectra are identical upfield of 7.0 ppm. Regions where the native spectrum has intensity not appearing in the mononitrated spectrum are shaded with horizontal lines. Such shading is concentrated in two regions of area two at 7.38 and 7.14 ppm. These regions therefore are assigned to tyrosine-10, the ring present in the native BPTI but absent in the mononitrated derivative. Even though tyrosine-10 resonances are not resolved from other aromatic resonances at this pH, they can be located in this chemical modification difference spectrum. Their chemical shifts are indicated by inverted triangles in Figure 8. In Figure 2, regions where the mononitrated spectrum has intensity not appearing in the native spectrum are shaded with slanted lines. Such shading is concentrated in three regions of area one at 8.37, 7.66, and 7.57 ppm. At pH 4.8 the nitrotyrosine resonances appear as a re-

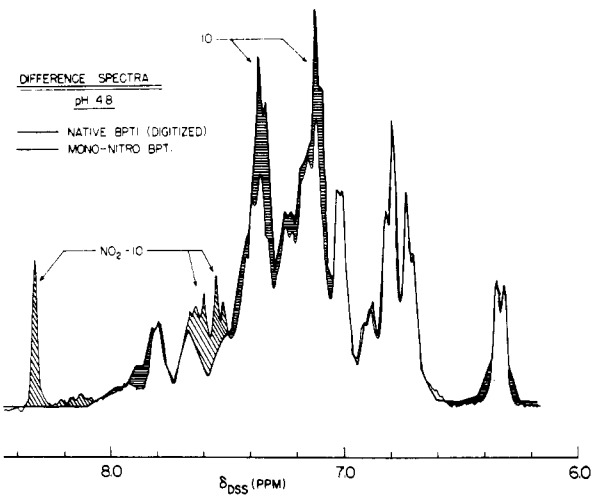

FIGURE 2: Superposition of the aromatic region (δ_{DSS} 8.4–6.2 ppm) of the 250-MHz NMR spectrum of preexchanged native and mononitrated BPTI; [protein], $\simeq 2.5 \times 10^{-3}$ *M* in 15 m*M* acetate buffer, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O (pH 4.8); $\simeq$300 scans using correlation spectroscopy method.

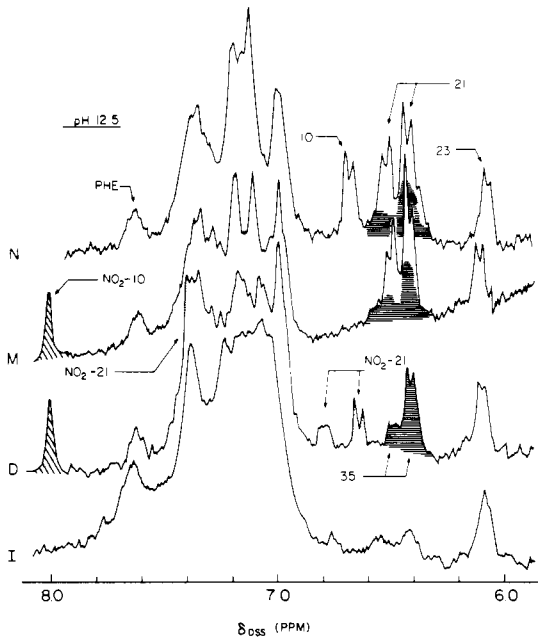

FIGURE 3: Aromatic region (δ_{DSS} 8.1–5.9 ppm) of the 250-MHz NMR spectrum of preexchanged BPTI derivatives; N, native; M, mononitrated; D, dinitrated; I, iodinated (see Table I for details); [protein], $\simeq 2.5 \times 10^{-3}$ *M* in 15 m*M* potassium phosphate buffer, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O (pH 12.50); $\simeq$300 scans using correlation spectroscopy method.

solved singlet and two closely spaced resolved doublets. These regions therefore are assigned to nitrotyrosine-10. Their chemical shifts are indicated by circles in Figure 8.

In Figure 3 the pH 12.5 spectra of the four derivatives in Table I are aligned. Resonances appearing outside the phenylalanine region are shaded according to the combinations of spectra in which they appear. Resonances occurring exclusively in the spectrum of dinitrated BPTI are shaded with dots, resonances associated with both nitrated derivatives are shaded with slanted lines, and resonances associated with all except the iodinated derivative are shaded with horizontal lines. The top spectrum is that of the native inhibitor. Comparing it with the spectrum of the mononitrated inhibitor directly below it, nitration causes the disappearance of an upfield doublet of area two at 6.71 ppm and the new appearance of a downfield singlet of area one at 8.05 ppm. On the basis of Table II, these resonances therefore are assigned to tyrosine-10 and nitrotyrosine-10, respectively. Similarly, a comparison of the spectra of the mono- and dinitrated derivatives reveals the loss of both tyrosine-21 doublets with a total area corresponding to four protons and the new appearance of each of the three resonances of the nitrated ring. Following iodination, resonances assigned to tyrosines-10, -21, and -35 disappear, with the remaining doublet assigned to the chemically inert tyrosine-23. The resolved downfield resonance occurring in all four spectra at 7.74 ppm is assigned to phenylalanine. It is not assigned to tyrosine-23 since it cannot be titrated, whereas the tyrosine doublet can be titrated. The above results reveal that the resonance of area one downfield from 7.5 ppm accounts for one phenylalanine proton and that the region of area 11 upfield from 6.9 ppm accounts for 11 tyrosine protons. The phenylalanine region therefore contains the resonances associated with the remaining 19 phenylalanine and five tyrosine protons.

Figures 2 and 3 illustrated comparisons of spectra of different derivatives at the same pH. Comparisons also can be made between samples of the same derivative examined at different pH's. Considering first native BPTI, no changes occur in the aromatic region on raising the pH from 4.8 to 8.5. On raising the pH from 8.5 to 13, numerous resonances titrate upfield with tyrosine-like p*K*'s. The results suggest that all spectral changes occurring in this pH interval can be associated with the 16 protons of BPTI's four titratable tyrosines. Many of the spectra of the basic titration of native BPTI were presented earlier (Karplus et al., 1973).

Figure 4 illustrates the acidic titration of mononitrated BPTI. In contrast to the results of native BPTI where no aromatic resonances titrated at pH's below 8.5, three resonances of area one titrate in Figure 4. These resonances, which are shaded when resolved downfield of the phenylalanine region, are assigned to nitrotyrosine-10 on the basis of their nitrotyrosine-like p*K*'s. At pH 4.68, the assignment in the bottom of Figure 4 is identical to that already obtained in Figure 2 by the chemical modification technique. During the basic titration of this derivative, the nitrotyrosine-10 resonances maintain a constant chemical shift. All spectral changes occurring above pH 8.5 can be associated with the 12 protons of the derivative's three titratable tyrosines.

Figure 5 illustrates the titration of the dinitrated derivative from pH 4.8 to 12.5. The shading of the pH 12.50 spectrum is duplicated from Figure 3, with shadings of the pH 8.43, 6.97, 6.08, and 4.83 spectra derived from similar chemical modification alignments. The pH 8.43 and 9.32 spectra are identical. Resonances already assigned to nitrotyrosines-10 and -21 shift upfield on raising the pH from 4.8 to 8.4 but do not shift position at higher pH's. Conversely, resonances assigned to tyrosines-23 and -35 maintain constant chemical shifts at pH's below 9.3, but shift upfield on raising the pH above 9.3 The resolved downfield phenylalanine resonance is not titratable.

In obtaining the assignments of the aromatic tyrosine resonances of BPTI, an important aspect of the nitrated derivatives was their significantly lower p*K*'s. The best example is provided by considering the complex multiplet resulting from the overlap of resonances of the 21 and 35 rings. At pH 7, the spectrum of native BPTI seen in Figure 9 contains a multiplet of area seven centered at 6.8 ppm. It con-

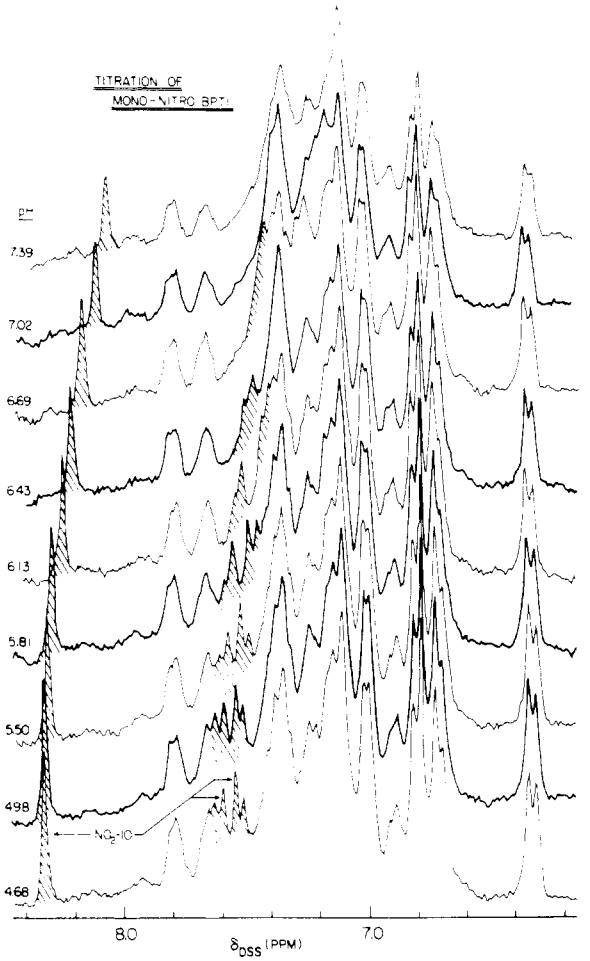

FIGURE 4: Aromatic region (δ_{DSS} 8.4–6.2 ppm) of the 250-MHz NMR spectrum of preexchanged mononitrated BPTI; [mononitrated BPTI], $\simeq 2.5 \times 10^{-3}$ *M* in combination buffer of 15 m*M* acetate and 15 m*M* Tris, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O, pH as indicated; $\simeq$300 scans using correlation spectroscopy method.

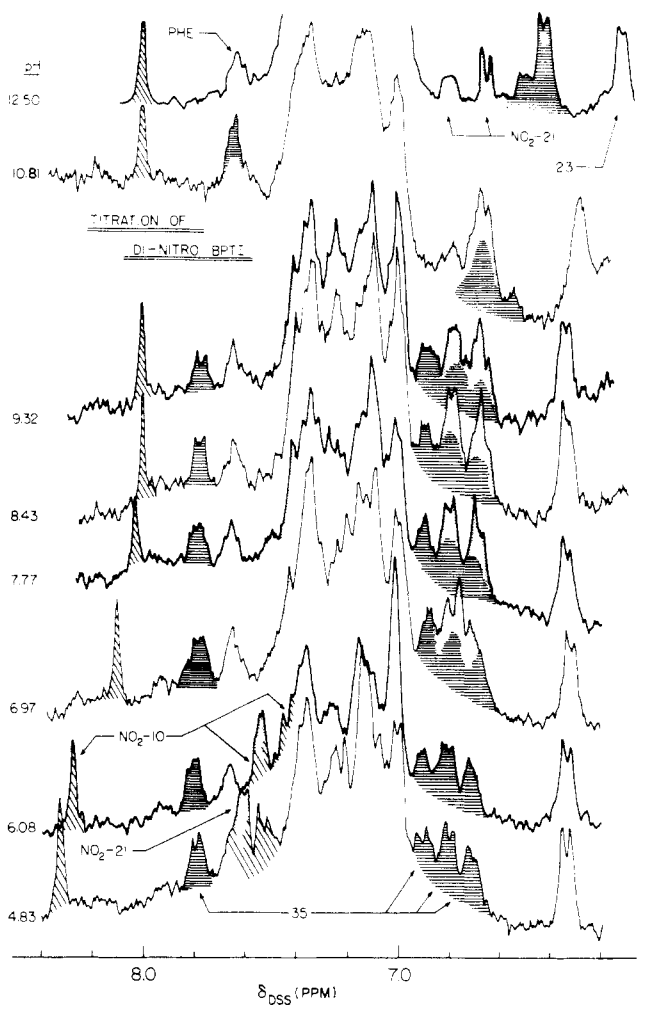

FIGURE 5: Aromatic region (δ_{DSS}, 8.4–6.2 ppm) of the 250-MHz NMR spectrum of preexchanged dinitrated BPTI; [dinitrated BPTI], $\simeq 2.5 \times 10^{-3}$ *M* in combination buffer of 15 m*M* acetate, 15 m*M* Tris, and 15 m*M* potassium phosphate, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O, pH as indicated; $\simeq$300 scans using correlation spectroscopy method.

sists of overlapping resonances from four tyrosine-21 protons and three tyrosine-35 protons. Because both rings have tyrosine-like p*K*'s, the resonances titrate together, preventing resolution of the overlapping peaks in the unmodified inhibitor. If the native inhibitor is dinitrated, the pH 7 spectrum contains a complex pattern of area five centered at 6.8 ppm as seen in Figure 5. It consists of the overlap of resonances from two nitrotyrosine-21 protons and three tyrosine-35 protons. Now, however, because nitrotyrosine and tyrosine rings have distinctly different p*K*'s, the nitrotyrosine components of the composite multiplet can be resolved by shifting the tyrosine resonances upfield at higher pH's, whereas the tyrosine components can be resolved by shifting the nitrotyrosine resonances downfield at lower pH's. In particular, at pH 4.8 tyrosine-35 is resolved into four separate doublets of area one (Figure 5).

Spectra of the same derivative at different pH's were subtracted in a pairwise manner to produce difference spectra similar in character to Figure 2. For example, the pH 7.02 and pH 4.68 spectra of mononitrated BPTI, presented separately in Figure 4, were superimposed following scaling of the pH 7.02 spectrum to give identical intensities for the resolved upfield doublet at 6.37 ppm. As seen in Figure 4, the phenylalanine region of the pH 4.68 spectrum contains no nitrotyrosine-10 resonances. In the difference spectrum, the phenylalanine region of the pH 7.02 spectrum contained two regions of extra intensity not present in the pH 4.68 spectrum. These corresponded to resonances of area one at 7.40 and 7.23 ppm, thereby determining the positions of the unresolved H-5 and H-6 resonances of nitrotyrosine-10 in the pH 7.02 spectrum. These chemical shifts are indicated by upright triangles in Figure 8.

Additional assignment information can be obtained from double resonance experiments. Figure 6 consists of 250-MHz spectra of the tyrosine region (upfield of 6.9 ppm) of the native inhibitor at pH 12.5 obtained while systematically irradiating downfield throughout the phenylalanine region at 10-Hz intervals. The upfield doublet of tyrosine-10 at 6.71 ppm is decoupled when irradiating in the phenylalanine region at 7.18 ppm. Since this is the only irradiation frequency giving decoupling, the resonances of both unresolved protons must occur at 7.18 ppm forming a second doublet of area two. Similarly, the upfield doublet of area two of tyrosine-23 at 6.15 ppm is coupled to a downfield doublet of area two at 7.06 ppm. The decoupling of both upfield doublets is accompanied by a decrease in intensity resulting from a negative nuclear Overhauser enhancement. This experiment was repeated at 100 MHz with identical results except for the lack of a nuclear Overhauser effect.

Similar experiments were performed at 250 MHz on na-

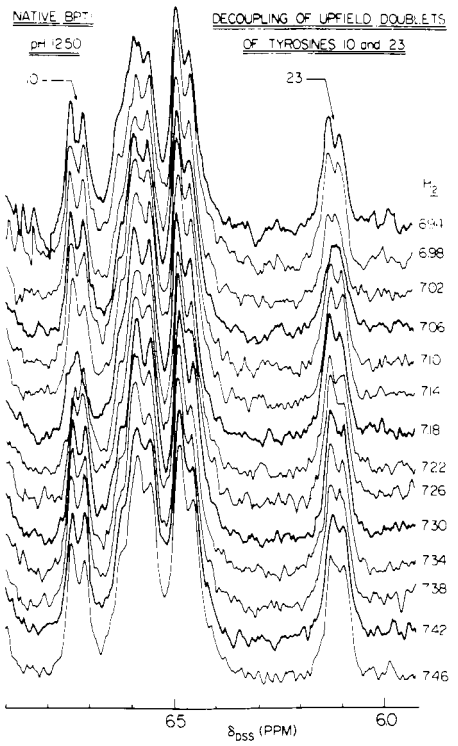

FIGURE 6: Upfield tyrosine region (δ_{DSS} 6.9–5.9 ppm) of the 250-MHz NMR spectrum of preexchanged native BPTI; decoupling irradiation (H_2) at 10-Hz intervals in downfield phenylalanine region (δ_{DSS} 7.5–6.9 ppm) as indicated; [BPTI], $\simeq 2.5 \times 10^{-3}$ *M* in 15 m*M* potassium phosphate buffer, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O (pH 12.50); $\simeq$50 scans using correlation spectroscopy method.

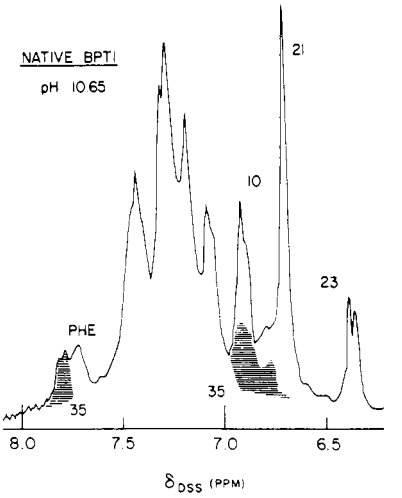

FIGURE 7: Aromatic region (δ_{DSS} 8.1–6.2 ppm) of the 250-MHz NMR spectrum of preexchanged native BPTI: [BPTI], $\simeq 2.5 \times 10^{-3}$ *M* in 15 m*M* potassium phosphate buffer, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O (pH 10.65); $\simeq$200 scans using correlation spectroscopy method.

tive BPTI at pH 8.5 and 4.8. The spectra without decoupling are identical to the pH 7.0 spectrum in Figure 9. In both cases, the resolved downfield doublet of tyrosine-35 at 7.84 ppm was decoupled when irradiating at 6.84 ppm, the position of the middle of the three upfield tyrosine-35 resonances. Also, the 3,5 doublet of tyrosine-23 was decoupled when irradiating at 7.19 ppm, thereby locating the corresponding unresolved 2,6 doublet. Identical results were obtained at 250 MHz on the pH 4.83 dinitrated sample. Moreover, since the three upfield tyrosine-35 doublets are resolved in this derivative, as seen in the bottom of Figure 5, it therefore was possible to observe them while irradiating downfield at 7.84 ppm. Only the center doublet at 6.84 ppm was decoupled. Thus the coupled tyrosine-35 doublets at 7.84 and 6.84 ppm are associated with protons located on one side of the ring, with the doublets at 6.95 and 6.75 ppm corresponding to the other side. This result is indicated in Figure 8 by the $^2/_3$ and $^5/_6$ notations.

The occurrence of a negative nuclear Overhauser enhancement within the phenylalanine region on the irradiation of a resolved tyrosine resonance provides an additional method for locating the unresolved tyrosine resonances. For example, two spectra were obtained of native BPTI at pH 8.50 with decoupling irradiation at 6.39 ppm, the frequency of the resolved upfield tyrosine 23 doublet, and at 6.43 ppm, a control frequency where no resonances occur. Although the decoupling of the downfield tyrosine-23 doublet was not visible due to its unresolved nature, the decoupling was accompanied by a negative nuclear Overhauser enhancement. Thus computer subtraction of the control spectrum from the decoupled spectrum resulted in a difference spectrum containing a single negative peak at 7.23 ppm. This therefore is the position of the downfield tyrosine-23 doublet, agreeing with the result of 7.19 ppm reported above.

Additional information can be obtained by consideration of intensity patterns of resolved doublets in single spectra. For example, in the pH 12.5 spectrum of native BPTI at the top of Figure 3, examination of the two lines comprising the resolved tyrosine-10 doublet at 6.74 ppm reveals that the line of greater intensity is on the downfield side of the doublet. This indicates that the remaining tyrosine-10 resonances occur downfield of 6.74 ppm with resonances upfield of this point belonging to other rings, in agreement with results obtained by other methods. Furthermore, when two resolved doublets are coupled to each other and separated by a small chemical shift, the multiplet of four lines should exhibit AB character, with the intensities of the inner two lines growing at the expense of the outer two lines. Such behavior can be seen in the spectrum of the mononitrated derivative in Figure 2, containing closely spaced nitrotyrosine-10 doublets centered at 7.61 ppm and tyrosine-21 doublets centered at 6.80 ppm. In the pH 4.83 spectrum of dinitrated BPTI at the bottom of Figure 5, tyrosine-35 is resolved into four doublets of area one. Since the downfield line of the doublet at 6.84 ppm has greater intensity than the upfield line, this resonance is coupled to one of the two tyrosine-35 doublets occurring on its downfield side. Its lack of strong AB character indicates that it is coupled to the resonance at 7.83 ppm rather than that at 6.95 ppm only 0.11 ppm away. This agrees with the results of the decoupling experiments. Finally, when coupled resonances directly overlap, the doublet character is lost giving a singlet with AA′ character. Inspection of Figure 8 reveals that superposition of the coupled H-5 and H-6 resonances of nitrotyrosine-21 occur at pH 6.08, with superposition of the ortho and meta resonances of the unmodified ring occurring at pH 10.65. The former overlap results in a singlet of area two at 7.05 ppm in the pH 6.08 spectrum of Figure 5. The latter results in a singlet of area 4 at 6.70 ppm in Figure 7. In the pH 12.50 spectrum of dinitrated BPTI in Figure 5, two of the three upfield resonances of tyrosine-35 overlap at 6.47 ppm to form a doublet of area two. Since the superposition of the H-5 and H-6 resonances would result in a singlet with AA′ character, this doublet must consist of resonances with

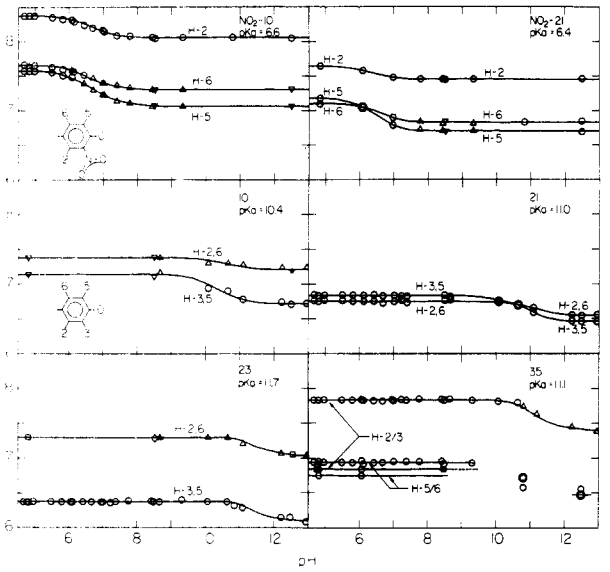

FIGURE 8: Chemical shifts of all tyrosine and nitrotyrosine resonances in the aromatic region (δ_{DSS} 9.0–6.0) of the 250-MHz NMR spectrum of preexchanged native, mononitrated, and dinitrated BPTI as a function of pH (see Table II for samples); [protein], $\simeq 2.5 \times 10^{-3}$ *M* in combination buffer of 15 m*M* acetate, 15 m*M* Tris, and 15 m*M* potassium phosphate; 0.1 *M* KCl and 0.1 m*M* DSS in D_2O. All chemical shifts for tyrosine and nitrotyrosine resonances (except H-2 singlet of nitrotyrosine) correspond to the midpoint of the observed doublets. pK_a's are the calculated non-linear least-squares fits to single proton titration curves (see Results for details). Curves are hand drawn. Symbols indicating method of determination of chemical shifts are: (○) resolved resonances, (▽) chemical modification difference spectra, (△) titration difference spectra, (□) double resonance decoupling, (◐) both titration and chemical modification difference spectra, (★) both chemical modification difference spectra and decoupling, and (◇) both negative NOE difference spectra and decoupling. Symbols indicating the overlap of two resonances are pairs of concentric circles or hexagons.

identical chemical shifts arising from protons on opposite sides of the ring, each with the same coupling to a proton on the same side of the ring. The summary of tyrosine-35 results in Figure 8 contains a dotted line to illustrate that the upfield doublet of the ⅔ pair occurring at 6.84 ppm at pH 4.8 titrates to 6.47 ppm at pH 12.50.

The complete results are summarized in Figure 8. The tyrosine-10 and nitrotyrosine-21 rings occur in only one derivative apiece. The chemical shifts of the ring protons in the remaining tyrosines and in nitrotyrosine-10 have been determined in several different derivatives. For each particular resonance, the chemical shifts observed in the different derivatives have been plotted in a single smooth titration curve. In all cases where a particular resonance was located by different techniques or occurred in several derivatives examined at the same pH, the results agreed within ±0.02 ppm with the average chemical shift reported in the figure. The composite data sets were used for the titration fits, with parameters summarized in Table III and compared in Table IV. In the case of the H-2 resonance of nitrotyrosine-10, separate fits were made for the individual data sets from mononitrated and dinitrated BPTI. As seen in Table III, all four parameters agree when comparing the results of the individual derivatives with each other or with the results obtained for the composite data set. The p*K*'s summarized in Figure 8 are the average of the p*K*'s of different resonances associated with the same ring.

Tyrosine and nitrotyrosine resonances are assigned to ring positions in two ways. The H-2 proton of nitrotyrosine

Table V: Titration of Tyrosine-35—Changes in Chemical Shift.

Ring Side	Comment[b]	δpH 4.83[a]	δpH 12.50[a]	$\Delta\delta$
2/3		7.83	7.42[c]	0.41
2/3		6.84	6.47	0.37
5/6	C	6.95	6.47	0.48
5/6	C	6.75	6.55	0.20
5/6	N	6.95	6.55	0.40
5/6	N	6.75	6.47	0.28

[a] Chemical shift in ppm from DSS. [b] C, resonances chosen as crossing; N, resonances chosen as noncrossing. [c] Value determined by interpolation between pH 12.22 and pH 12.95 results.

is distinguished on the basis of its singlet nature. All other protons are assigned to ortho and meta positions on the basis of their relative changes in chemical shifts during titration. As discussed earlier, the ortho protons of tyrosine peptides typically titrate 0.12 ppm further during titration than the protons meta to the hydroxyl group. Hence since the upfield doublet of tyrosine-23 titrates 0.12 ppm further than the downfield doublet, these resonances are assigned to the ortho and meta protons, respectively. The two doublets of unmodified and nitrated tyrosine-10 were assigned to ring positions by similar comparisons.

The behavior of the native and nitrated forms of tyrosine-21 is more complex than that observed in tyrosine-10 due to the overlap of resonances at intermediate pH's in the titration. For example, the two resonances of unmodified tyrosine-21 overlap at pH 10.65. Thus one must determine whether or not crossover occurs, that is whether the downfield resonance at low pH corresponds to the downfield or upfield resonance at high pH. Separate fits were made for the noncrossing and crossing treatments. The ortho/meta comparisons listed in Table IV suggest that crossover is more likely. First of all, the crossover treatment gives closer agreement of p*K*'s and Hill coefficients. Secondly, when the resonances are treated as crossing they differ in the magnitude of change in chemical shift occurring on titration, whereas they do not differ in the noncrossing case. The former behavior is more typical of tyrosine peptides. Finally, the average standard deviation of fits is lower in the crossing case. Thus crossover is more likely, with the resonances finally assigned to ortho and meta positions on the basis of relative chemical shift changes. At neutral pH, the two tyrosine-21 doublets therefore occur in an order which is inverted with respect to that observed in model compounds. A similar analysis suggests that crossover also occurs in the nitrated form of tyrosine-21.

The behavior of tyrosine-35 is too complex to permit assignment to ortho and meta positions on the basis of the observed changes in chemical shift occurring during titration. The tyrosine-35 behavior, summarized in Table V, will be considered in the Discussion section, together with a model to explain the crossover behavior of tyrosine-21.

The results and assignments are summarized in Figure 8. It now is possible to examine a single spectrum and observe the diversity of behavior exhibited by the inhibitor's four tyrosines. Figure 9 presents the pH 7 spectrum of native BPTI. The tyrosine-35 resonances appear as four doublets of area one, while the remaining three tyrosines each give two doublets of area two. Of these latter rings, the ortho resonances of tyrosines-10 and -23 occur upfield of their corresponding meta resonances as is observed in model compounds, but the ortho and meta resonances of tyrosine-

21 occur in inverted order. Coupled resonances are separated by less than 0.24 ppm in tyrosines-10, -21, and the 5/6 pair of tyrosine-35, but are separated by more than 0.86 ppm in tyrosine-23 and the 2/3 pair of tyrosine-35.

Discussion

This section first discusses the validity of two assumptions used in earlier analysis of the results, namely the completeness of exchange of buried NH's and the independence of the behavior of the aromatic rings. Secondly, specific aspects of the behavior of BPTI's tyrosines will be considered, such as the immobility of tyrosine-35, the asymmetry of inductive effects experienced by tyrosine-35 resonances on deprotonation, and the crossover of tyrosine-21 resonances. Then comparisons will be made between the nuclear magnetic resonance results in this manuscript and results obtained by other experimental techniques. Finally, the BPTI results will be used to evaluate the relative strengths and weaknesses of different tyrosine assignment techniques.

Although BPTI is small, it is very stable and possesses an interior sufficiently protected to contain at least nine buried NH's with half-times of exchange greater than 4 months at pH 7 (Karplus et al., 1973; Masson and Wüthrich, 1973). All NH's with resolved resonances downfield of 7.9 ppm can be completely exchanged with D_2O solvent by either of the techniques described in the Experimental Section. Both techniques denature BPTI to the extent that the four phenylalanines and the four tyrosines have equivalent magnetic environments. However, since the disulfide bridges remain intact with both procedures, some exchangeable protons with resonances upfield of 7.9 ppm might remain buried in the molecule. The results reported above suggest that this possibility is slight. First, the spectra of derivatives exchanged by different procedures are identical. For both methods to give precisely the same incomplete exchange would be a coincidence. Secondly, the area measurements were consistent with 36 aromatic protons, within an error of approximately ±2 protons, with no indication of the presence of resonances of exchangeable protons.

Comparisons of spectra of chemically modified derivatives are possible if the NMR behavior of a particular aromatic ring is independent of chemical modifications occurring at other aromatic rings. This independence, assumed in making the above assignments, can be demonstrated explicitly for the tyrosines in BPTI. For example, the results in Figure 3 demonstrate the lack of any perturbation in the environment of the remaining tyrosines upon chemical modification of one or more tyrosines at pH 12.5. Tyrosine-23 is independent of each of the three other tyrosines, since the chemical shift experienced by two of its protons is invariant on iodination of ring 35 and either iodination or nitration of rings 10 and 21. Similarly, the resonances of three protons of tyrosine-35 are unchanged on nitration of tyrosine-10 or -21. Finally all four protons of tyrosine-21 are uninfluenced by nitration of 10 and at least one proton of nitrotyrosine-10 is not influenced by nitration of -21. The appropriate substitution of nitrotyrosine(s) or iodotyrosine(s) accounts for all the spectral changes occurring in Figure 3. Possible structural or magnetic changes resulting from the introduction of the nitro or iodo groups are either too small or too localized to influence the other aromatic rings. Similar behavior exists at each of the other four pH's listed in Table II where different derivatives were examined at the same pH.

In an analogous fashion, the pH titration of a given tyrosine is straightforward only if the chemical shift of the aromatic ring's resonances change as a result of its own titration and do not reflect changes in the state of ionization of other groups which titrate over the same pH range. With the chemical substitutions, at most three residues were modified. This is not the case during titrations however, since BPTI and its derivatives contain 20 titratable groups. Nevertheless, all of the tyrosine chemical shift titration data were consistent with simple ionizations without the need to invoke interactions with other ionizable groups.

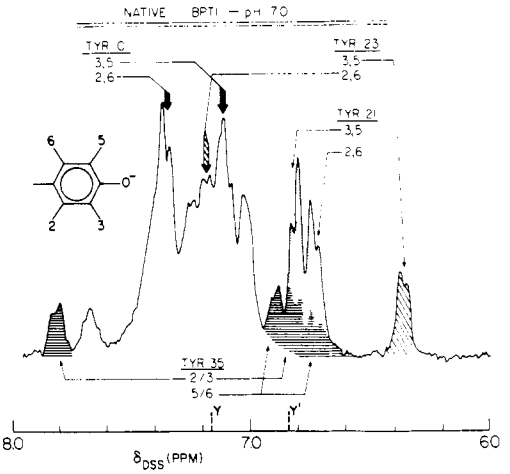

FIGURE 9: Aromatic region (δ_{DSS} 8.0–6.0 ppm) of the 250-MHz NMR spectrum of preexchanged native BPTI; [BPTI], $\simeq 2.5 \times 10^{-3}$ *M* in 15 m*M* Tris buffer, 0.1 *M* KCl, 0.1 m*M* DSS, and D_2O (pH 7.0); $\simeq$300 scans using correlation spectroscopy method. Y and Y′ indicate the chemical shifts of resonances corresponding to the 2,6 and 3,5 protons, respectively, of 10 m*M* *N*-acetyl-L-tyrosineamide in the same buffer at pH 7.0.

Several specific interactions between titratable groups can be ruled out explicitly. For example, the identity of the spectra of the native inhibitor at pH 4.8 and 8.5 indicate that the protonated forms of the four tyrosines are independent of any carboxyl titration in this pH interval. Similarly, the spectra of mononitrated BPTI in Figure 4 demonstrate that the protonated forms of tyrosine-21, -23, and -35 are independent of the titration of nitrotyrosine-10. At pH's above 8.5, the pH-independence of the chemical shifts of the nitrotyrosine resonances indicates that the deprotonated form of nitrotyrosine-10 is independent of the titrations of the other three tyrosines, and similarly for the dinitrated derivative in Figure 5. The independence of the tyrosine rings on chemical modification and titration permits one to combine the chemical shifts measured in different derivatives in the same titration curves in Figure 8.

The resolution of the tyrosine-35 resonances into doublets of area one demonstrates that this ring is immobile in BPTI on the NMR time scale at 25° in the pH interval between pH 5 and 9. Since the resonances of the H-3 and H-5 ortho protons or the H-2 and H-6 meta protons do not have the same chemical shift, they must be experiencing inequivalent environments on opposite sides of the tyrosine ring. This can occur only if the ring is relatively immobilized in the protein structure. On the other hand, when the meta protons and the ortho protons appear as two doublets of area two, as with the remaining three tyrosines, one cannot conclusively determine whether or not the ring is rotating. The ring could be immobilized with environments on opposite sides of the ring equivalent by coincidence, or the ring could be rotating freely such that any nonequivalence would be averaged out. Since it is a priori more likely in a protein that en-

vironments on opposite sides of a ring will be unequal, the existence of equivalent ortho and meta protons in tyrosine-10, -21 and -23 suggests that these rings are rotating freely on the NMR time scale at 25° over the pH range from 5 to 13. Considering the ring protons of tyrosine 35 between pH 5 and 9, the protons with resonances at 7.83 and at 6.84 ppm are coupled and correspond to protons on the same side of the ring. The resonances at 6.95 and at 6.75 ppm must therefore correspond to protons on the other side of the tyrosine ring. Because of the unique downfield shift of the resonance at 7.83 ppm, the relative separations of the two ortho proton resonances and of the two meta proton resonances must differ by a factor of ten (1 ppm vs. 0.1 ppm) in this pH range. In order to observe the more closely spaced pair, the slow exchange relationship ($\tau\Delta\omega > 1$) indicates that tyrosine-35 must rotate at a rate ($1/\tau$) less than 160 times/sec. If one were to assume that environmental contributions to chemical shifts of protons on opposite sides of the rings of tyrosine-10, -21, and -23 differ by at least 0.1 ppm for the ortho or meta pair of protons, the fast exchange relationship ($\tau\Delta\omega < 1$) would lead to the conclusion that these three rings rotate faster than 160 times/sec. Preliminary calculations (Gelin and Karplus, 1975) of the relative barriers to rotation for BPTI's tyrosines suggest that tyrosine-10, -21, and -23 rotate at rates $\geq 1.3 \times 10^4$ times/sec and that tyrosine-35 rotates at a rate equal to 1.4×10^{-4} times/sec, consistent with the experimental limits described above. The experimental results do not permit discussion of phenylalanine mobilities, since the only resolved phenylalanine resonance of area one, that occurring at 7.74 ppm, may be associated with a H-4 proton of a phenylalanine ring.

Although the unmodified tyrosine-10 and -21 rings are presumed relatively free to rotate, the substitution of a nitro group for a hydrogen conceivably could introduce new steric restrictions to rotation. However, the rotational freedom of nitrotyrosine-10 and -21 cannot be determined on the basis of their appearance as three resonances of area one at all pH's between pH 5 and 13 since the corresponding three protons are intrinsically inequivalent, giving three resonances in model nitrotyrosine peptide spectra where there is unrestricted rotation.

The dinitrated derivative has not yet been studied at enough pH's to permit the construction of complete titration curves for the three upfield resonances of tyrosine-35 in Figure 8. Although the pH 4.83 and pH 12.50 chemical shifts are known for both resonances of the 2/3 pair as summarized in the top of Table V, it is not known whether crossover occurs for the two resonances of the 5/6 pair. Both cases are included in Table V. If crossover occurs, then the changes in chemical shift on titration consist of one change of 0.5 ppm, two of 0.4 ppm, and one of 0.2 ppm. If crossover does not occur, then the chemical shift changes consist of three values of 0.4 ppm and one of 0.3 ppm. In either case, the results demonstrate that one cannot assign two resonances to ortho protons experiencing an identical change in chemical shift with two resonances assigned to meta protons experiencing a smaller but identical change. Thus the inductive effects resulting from deprotonation are not distributed symmetrically along both sides of the ring. Either the inductive effects are modulated by the different environments on opposite sides of the immobile ring, or a change in environments accompanies titration of tyrosine-35.

The titration behavior of the tyrosine-35 resonances may be due to an increase in the rate of ring rotation accompanying the high pH loosening of the BPTI structure. Comparison of the spectra of native BPTI before and after exposure to pH 12.5 for 2 hr revealed that the half-time of exchange with D_2O of about a half-dozen buried NH's changes from greater than 4×10^3 hr at pH 7 to less than 1 hr at pH 12.5. Furthermore, on titrating from pH 10.65 to 11.10, two NH resonances displayed broadening caused by the onset of conformational exchange (Karplus et al., 1973). This suggests that the inhibitor is less rigid at high pH. If the rotation rate of the tyrosine-35 ring increased from less than 160 times/sec to a rate intermediate between 160 and 1600 times/sec, τ would be in the fast exchange limit for resonances separated by 0.1 ppm at 250 MHz but would still be in the slow exchange limit for resonances separated by 1.0 ppm. For tyrosine-35, this would result in a spectrum containing two widely spaced doublets of area one and a third doublet of area two, as observed in the pH 12.5 spectrum in Figure 5. Furthermore, the two resonances associated with a pair of nonequivalent protons on opposite sides of the ring would titrate by different amounts. Both would experience identical inductive effects, but the two resonances would approach a motionally averaged equivalent chemical shift from opposite directions.

The occurrence of crossover in both unmodified and nitrated 21 can be explained by a variety of models. A likely explanation is that during rotation of the tyrosine ring, each of the meta protons spends some time in a highly shielded environment in the protein. Inspection of Table III indicates that the ortho protons in both forms of tyrosine-21 have chemical shifts similar to those observed in model compounds between pH 5 and 13. The meta protons, however, are consistently shifted upfield, with differences relative to model compounds ranging from 0.25 to 0.44 ppm in the same pH interval. This behavior would result if the meta environment on one side of the ring contains a group causing an upfield shift. Then the equivalent average environment experienced by meta protons on the rotating unmodified ring would shift their doublet upfield as observed. If this environment and rotation were conserved following nitration of the ring, both the H-2 and H-6 resonances of nitrotyrosine would continue to be shifted upfield, as also is observed.

This manuscript will not attempt to interpret specific changes in chemical shift occurring during titration in terms of the known crystal structure. However, because all tyrosine resonances have been located and assigned, it is appropriate to estimate the contributions of ring currents to the observed shifts. The results of ring current calculations (Sternlicht and Wilson, 1967) for each of the 36 aromatic protons in BPTI predict that the net ring current contributions to chemical shifts at a particular location would exceed 0.1 ppm only for three phenylalanine protons, with all shifts upfield and ranging from 0.1 to 0.3 ppm in magnitude. These results are not able to explain several observed shifts of BPTI resonances relative to those in model compounds, such as the downfield shift of the phenylalanine resonance at 7.74 ppm, the downfield shift of the lone tyrosine-35 doublet, or the upfield shifts of the 2,6 doublet of tyrosine-21 and the 3,5 doublet of tyrosine-23. Assuming that these calculations accurately predict ring current contributions to chemical shifts, then either the solution and crystal structures of native BPTI are different or the observed shifts are dominated by the net sum of effects other than ring currents. Such effects could include the chemical shift anisotropies of groups other than aromatic rings, such as disulfide bridges and carbonyl groups, or the steric polar-

ization (Cheney, 1968) resulting from protons in van der Waals contact with each other.

The specific p*K*'s observed for BPTI's tyrosines agree with qualitative results observed by other techniques. First, uv–visible titration studies (Sherman and Kassell, 1968) of native and iodinated BPTI revealed that the one tyrosine with a uniquely high pK was the chemically inert tyrosine-23, agreeing with the results in Figure 8. Secondly, it is not surprising that tyrosine-10, the most readily nitrated ring, has the lowest p*K* since nitration requires the phenolate form of tyrosine (Bruice et al., 1968) and nitration reactions were performed at pH 8.5 where tyrosine-10 has a higher percentage of this form than the other tyrosines. For tyrosine-10, the occurrence of an aromatic p*K* closest to the free amino acid value and the relative ease of nitration both reflect the relatively exposed position of this residue in the crystal structure (Deisenhofer and Steigemann, 1974). The variety of p*K*'s, chemical shifts, and mobilities of the tyrosine residues in BPTI, in agreement with the heterogeneity of their behavior in optical spectra (Brandts and Kaplan, 1973) and chemical modification studies (Meloun et al., 1968b; Sherman and Kassell, 1968), indicate the variety of their chemical environments within this small protein. Moreover, because the tyrosines are modified and titrated independently of each other, the NMR results indicate that no two tyrosines have hydroxyl groups spatially adjacent in solution, consistent with features of the known crystal structure (Deisenhofer and Steigemann, 1974).

Having identified the resonances of the 16 tyrosine ring protons in BPTI by complementary use of a variety of methods giving a single consistent and complete assignment, it is possible to evaluate the relative strengths and weaknesses of different tyrosine assignment techniques. For example, tyrosine assignments should not be based on comparisons of areas and chemical shifts with those observed in model peptides. Once tyrosines are imbedded in a protein matrix, even a protein as small as BPTI, they exhibit a variety of behavior different from that observed in model compounds such as *N*-acetyl-L-tyrosineamide. In model peptide spectra, tyrosine ring resonances occur as two resonances of area two upfield from phenylalanine ring resonances. In BPTI, the aromatic resonances of particular tyrosines (at various pH's) appear as one singlet of area four, two doublets of area two, a doublet of area two and two doublets of area one, or as four doublets of area one. In unexchanged BPTI, resolved resonances of area one downfield of the phenylalanine region include resonances associated with a buried NH proton, a phenylalanine ring proton, and a tyrosine proton in a relatively immobilized ring. In general, resolved upfield doublets of area two need not be associated a priori with tyrosines, since such doublets have been observed for phenylalanine resonances in peptides containing two aromatic rings (Cohen, 1971; Balaram et al., 1972). Finally, the crossover of the meta and ortho proton resonances of tyrosine-21 at pH 10.65 show that the upfield doublet of tyrosine-21 at pH 7 cannot be assigned to the ortho protons on the basis of the upfield chemical shift of tyrosine ortho protons in model peptides.

Decoupling is a useful technique for observing coupled resonances when restricted to the observation of the decoupling of resolved doublets not overlapping any other resonances, as was the case for the results reported above. However, the technique is not reliable when observing apparent decoupling in overlapping resonances. Because negative nuclear Overhauser enhancements can occur between spatially adjacent protons located on different aromatic rings as well as between adjacent protons on the same ring, irradiation in one region of the spectrum may cause changes in intensity in several other regions. Where resonances overlap, such intensity changes may lead to the apparent collapse of doublets when decoupling actually is not occurring. For similar reasons, negative nuclear Overhauser enhancements may be an ambiguous technique for locating coupled tyrosine resonances. However, all assignments must be consistent with the negative NOE's observed. Furthermore, in larger proteins in which the increased line widths preclude the observation of spin–spin coupling constants, the NOE may be essential in making the assignments (Campbell et al., 1974).

For *distinguishing* tyrosine resonances from other aromatic resonances, the most useful technique is titration at basic pH's since phenylalanine and tryptophan residues are not titratable and histidines titrate at acidic pH's. In nitrated derivatives, nitrotyrosine resonances can be located by titration at acidic pH's, although histidines titrate concurrently. In BPTI, the complete location of resonances associated with the 16 tyrosine and the six nitrotyrosine protons is facilitated by its lack of histidines and by its stability and solubility throughout the pH interval 2–13. For *assigning* tyrosine resonances to particular residues, the most useful technique is chemical modification, particularly the selective nitration of tyrosines. Nitration occurs only at tyrosine residues and free cysteine sulfhydryl groups, results in modification of only one tyrosine ortho position, and lowers a tyrosine ring's p*K* from 10 to 7, thereby permitting the isolation of purified derivatives by ion-exchange chromatography. It is much more difficult to prepare derivatives with selective iodination of tyrosines since iodination can occur at histidines, tryptophans, and free cysteines as well as tyrosines, either one or both ortho tyrosine positions may be modified, and monoiodination lowers a tyrosine ring's p*K* from 10 to only 8.5. Instead, it sometimes is easier to exhaustively iodinate a protein and search for tyrosines which are selectively inert to any modification. In BPTI, the absence of histidines, tryptophans, and free cysteines, the selective nitration of tyrosine-10 followed by nitration of tyrosine 21, and the selective inertness to iodination of tyrosine 23 make it possible to uniquely and completely assign all tyrosine resonances to particular residues without needing to identify the resonances of the iodinated tyrosine residues or to make inferences based on features of the crystal structure. After resonances are assigned to a particular residue, *further assignment of doublets to ortho and meta positions* is made by the comparison of relative magnitudes of change in chemical shift on pH titration. In BPTI, this permitted the complete ortho/meta assignment of resonances associated with tyrosine-10, -21, and -23 and nitrotyrosine-10 and -21. Such assignment was not possible for the four resolved tyrosine-35 resonances, but it was possible to assign them to opposite sides of the ring.by decoupling experiments.

It is useful to consider the revisions we have made to our earlier assignments (Karplus et al., 1973) in terms of the above evaluation of tyrosine assignment techniques. Chemical shifts in the former manuscript were measured with respect to an external standard of Me_6Si_2O, whereas chemical shifts here are reported from an internal standard of DSS. Subtracting 0.21 ppm from the earlier reported shifts, one obtains the following correspondence for native BPTI at pH 7 between labels in Figure 1 of the first manuscript and Figure 9 in this manuscript: peak "11", downfield tyrosine-35 resonance (7.83 ppm DSS); "12", resolved phenylala-

nine resonance (7.74 ppm); "V", peak comprised of phenylalanine resonances (7.03 ppm); and "IV", 3,5 doublet of tyrosine-23 (6.83 ppm). At higher pH's, "I" corresponds to the 3,5 doublet of tyrosine-10, "II-crossing" corresponds to the 2,6 doublet of tyrosine-21, and "III-crossing" to the 3,5 doublet of tyrosine-21. In addition, the "II-III" region includes intensity corresponding to three protons of tyrosine-35. The earlier manuscript contains a typographical error. The "II" and "III" labels should be interchanged in Table II to make them consistent with the discussion and Figures 1 and 2 in that manuscript.

Peak "11", a tyrosine doublet of area one, was erroneously assigned to an exchangeable proton. Its disappearance at basic pH's was attributed to exchange with solvent, and its titration upfield with increasing pH was attributed to a conformational change induced by neighboring titrating groups. Similar behavior was observed for peaks "1" and "5" which were correctly assigned to exchangeable protons. Peak "11" now is assigned to an aromatic ring proton since it fails to exchange with D_2O following either preexchange technique. Its disappearance at high pH results from titration upfield into the phenylalanine region. Its position at high pH has been determined in difference spectra as summarized in Figure 8. This peak is assigned to a tyrosine ring rather than to phenylalanine since it is titratable with a tyrosine-like pK and is coupled to another titratable resonance with a tyrosine-like pK. Additionally, both resonances disappear on iodination of three of BPTI's tyrosines. Although it might be possible for phenylalanine resonances to show apparent susceptibility to titration and iodination due to secondary environmental effects caused by a neighboring tyrosine, it is not reasonable to invoke secondary effects before first ruling out the primary explanation that peak "11" is associated directly with a tyrosine ring itself.

At pH 7, peak "V" earlier was assigned to the superposition of phenylalanine resonances and peak "I", as shown in Figure 2 of the earlier manuscript. As indicated in Figure 2 in this report, the correct position of peak "I", the 3,5 doublet of tyrosine-10, occurs at 7.15 ppm at low pH. The position of the doublet-like peak "V" at 7.03 ppm is identical in the spectra of native and mononitrated BPTI. Thus peak "I" must cross over peak "V" during titration. This crossover was not observed in the above studies since no native samples were studied at pH's between 8.67 and 10.08.[2] This revision to the earlier manuscript illustrates the usefulness of difference spectra between chemically modified derivatives and emphasizes the need for titrations at closely spaced pH intervals. At one point during our analysis, we believed that decoupling of peak "V" by irradiation at 7.4 ppm confirmed our earlier assignment. However, as discussed above, decoupling of an unresolved doublet cannot distinguish between tyrosine and phenylalanine resonances, since both phenylalanine and tyrosine resonances may occur as doublets.

At high pH's, the "II-III" region earlier was assigned to four tyrosine protons but now is assigned to seven tyrosine protons, including four from tyrosine-21 and three from tyrosine-35. The extra intensity not accounted for earlier is visible in the spectra of Figure 1 of that manuscript, but was not recognized as being real due to the noise and baseline distortions introduced in this spectral region during the early development of correlation spectroscopy. Peak "II-crossing", originally assigned to a 3,5 ortho doublet on the basis of chemical shifts observed in model compounds, now is assigned to an upfield-shifted 2,6 meta doublet on the basis of its smaller change in chemical shift occurring during titration as compared to the change observed in the other doublet associated with the same ring.

[2] The expected crossover has been observed in recent titrations which include spectra observed at pH's 9.05, 9.33, and 9.72.

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Added in Proof

Since submission of this manuscript, we received three other reports assigning aromatic resonances in BPTI. In a ^{13}C study of tyrosine titrations (Maurer et al., 1974), tyrosine pK's were reported with values significantly lower than those reported here. These values were obtained using only four samples between pH 8 and 11 with none at higher values, with pH's measured by an Ingold 405-M-5 electrode in solutions 0.2 M in Na^+. Our studies included samples at higher pH's, using a Radiometer GKS73041 HA-1 electrode calibrated at pH 13 with standard 0.1 M KOH solutions, with 0.1 M K^+ as the counterion in protein solutions. The results of a ^1H study of decoupling and basic titrations of native BPTI (Wagner and Wüthrich, 1975; Wüthrich and Wagner, 1975) differ from this manuscript with respect to which aromatic resonances are associated with phenylalanines and which with tyrosines. Since an attempt is made to account for all 16 tyrosine protons, these phenylalanine/tyrosine assignment differences occur in pairs. For example, at pH 7 the downfield tyrosine-35 resonance at 7.83 ppm was assigned to phenylalanine with its titratability not discussed, and the phenylalanine doublet-like peak at 7.03 ppm was assigned to include tyrosine resonances. These disagreements with the assignments in this manuscript occur at the same positions where we needed to revise our own earlier assignments of peaks "11" and "V", for the reasons outlined above.

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