Complete Tyrosine Assignments in the High-Field ^1H Nuclear Magnetic Resonance Spectrum of Bovine Pancreatic Trypsin Inhibitor Selectively Reduced and Carboxamidomethylated at Cystine 14–38†

Grayson H. Snyder,‡ Robert Rowan III,§ and Brian D. Sykes*¶

ABSTRACT: The low-field portions of the 250-MHz ^1H nuclear magnetic resonance spectra of native and chemically modified basic pancreatic trypsin inhibitor have been studied as a function of pH over the range pH 5–13. In derivatives selectively reduced and carboxamidomethylated at cystine 14–38, resonances associated with 15 of the 16 protons of the aromatic rings of the four tyrosines of the inhibitor have been located and assigned to specific tyrosyl residues. Titrations of pH yielded pK's for tyrosines 10, 21, 23, and 35 in the modified inhibitor of 9.9, 10.6, 11.6, and 11.0, respectively. Resonances associated with the three nitrotyrosine 10 protons of the mononitrated derivative and the six nitrotyrosine 10 and 21 protons of the dinitrated derivative have been similarly located, assigned, and titrated, yielding pK's for nitrotyrosines 10 and 21 of 6.5 and 6.4, respectively. Previously reported results for derivatives with cystine 14–38 intact have been revised on the basis of new data. Comparison of these revised results with the new data for derivatives with modified cystine 14–38 reveals no changes in pK's for any tyrosine or nitrotyrosine ring and no changes in chemical shift for resonances of nitrotyrosine 21 or tyrosines 21 and 23. However, modification of cystine 14–38 causes significant changes in chemical shifts of resonances of the nearby nitrotyrosine 10 and tyrosine 10 and 35 rings. Tyrosine 35 remains relatively immobile, rotating less than 1600 times/s at 25 °C for pH's in the range 5–13.

To facilitate an understanding of the processes by which proteins acquire their final three-dimensional shape following cellular synthesis as a linear polypeptide chain, attempts are being made to characterize structures which are intermediate between the fully denatured random-coil chain and the folded native state. Nuclear magnetic resonance has been used to examine the equilibrium population of structures under conditions of partially denaturing temperatures, pH's, or detergent concentrations. In studies of staphylococcal nuclease (Arata et al., 1973) and ribonuclease (Westmoreland and Matthews, 1973; Benz and Roberts, 1975a,b), it has been possible to observe sequential structural changes in different regions of the molecule by monitoring changes in resolved proton resonances assigned to specific histidines. Kinetic detection of intermediate structures under nonequilibrium conditions is also possible using NMR techniques, as in investigations of the refolding of thermally denatured ribonuclease following a sudden drop in temperature (A. Blum and R. L. Baldwin, private communication).

In proteins containing disulfide bridges it is possible to isolate intermediates with partial formation of these covalent cross-links, thereby potentially enabling comparison of nontransient species under physiological conditions. In the case of the bovine pancreatic trypsin inhibitor (BPTI),[1] a small protein of 58 amino acids whose single chain is cross-linked by three disulfides, it has been possible to prepare a series of eight derivatives containing 0, 1, 2, or 3 disulfides. Derivatives with one bridge have been prepared by quenching reoxidation of sulfhydryl groups in the completely reduced molecule (Creighton, 1974a,b). Derivatives with two bridges have been prepared by similar techniques (Creighton, 1975) or by selective reduction of the exposed cystine 14–38 in the native molecule (Kress and Laskowski, 1967; Meloun et al., 1968a; Liu and Meienhofer, 1968). The sequence and kinetics of formation of the disulfide intermediates have been reported (Creighton, 1975a–c).

Proton magnetic resonance techniques can be used to study such disulfide intermediates, provided that they are soluble at concentrations near 0.5 mM or greater and that resonances can be assigned to specific residues. In BPTI the tyrosine resonances associated with the 16 protons of the inhibitor's four tyrosines could be completely assigned to the individual tyrosine residues (Snyder et al., 1975) since each of the four tyrosines has a unique susceptibility to chemical modification in the native structure. This method of assignment can be applied to derivatives with varying numbers of disulfides if the tyrosine modifications are performed first and if derivatives with different tyrosine modifications then have identical susceptibilities to subsequent disulfide modifications. In the results to be described below, these criteria have been met in BPTI derivatives in which selective reduction and carboxamidomethylation of cystine 14–38 followed prior nitration or iodination at specific tyrosines. After assignments are made,

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts, 02138, and the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada. *Received October 21, 1975.* This work was supported in part by the National Institutes of Health (Grants GM-17190 and RR-00292 (NMR Facility for Biomedical Studies)) and the Medical Research Council of Canada Group on Protein Structure and Function.

‡ Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305.

§ Present address: Department of Chemistry, University of Maryland, College Park, Maryland 20740.

¶ Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

[1] Abbreviations used are: BPTI, basic pancreatic trypsin inhibitor; RCAM, reduction and carboxamidomethylation at cystine 14–38; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; CD, circular dichroism; ORD, optical rotatory dispersion.

the chemical shifts, pK's, and rates of rotation of each of the four tyrosines are compared in native BPTI and RCAM-unnitrated BPTI, demonstrating changes in the environments of tyrosines 10 and 35, the two tyrosines closest to cystine 14–38. Comparisons also are made with the derivative in which the remaining two cystines have been similarly reduced and blocked.

Experimental Section

BPTI was obtained as a gift from Farbenfabriken Bayer AG (Elberfeld, Germany). Low-molecular-weight impurities were removed by ultrafiltration at room temperature in an Amicon dialysis cell using a UM2 membrane and a 30 mM ammonium bicarbonate buffer at pH $\simeq$ 7.9. The protein solution was frozen at −30 °C and lyophilized to yield purified BPTI. Amino acid standard compounds were obtained from Sigma Chemical Co. and used without further purification. D_2O, DCl, and acidic-d_3 acid-d were obtained from Stohler Isotope Chemicals.

Some of the BPTI derivatives examined in this manuscript contain chemical modifications occurring both at tyrosines and at cystines. Whenever both types of modifications occur in the same molecule, the tyrosine modifications were performed first. Mononitrated and dinitrated BPTI were prepared as before (Snyder et al., 1975) using techniques based on modifications of an earlier study (Meloun et al., 1968b). The same batch of exhaustively iodinated BPTI prepared earlier (Snyder et al., 1975) was used in studies below. Native, mononitrated, dinitrated, and iodinated BPTI were then selectively reduced and carboxamidomethylated using a mild reaction procedure described earlier (Liu et al., 1971) which is known to give quantitative modification of cystine 14–38 in native BPTI. Native inhibitor also was completely reduced and carboxamidomethylated at all three cystines using the same procedure as above but performing reactions in 5 M guanidine-HCl and diluting the final solution tenfold prior to ultrafiltration to minimize destruction of the dialysis membrane. A similar preparation in 8 M urea was described earlier (Liu and Meienhofer, 1968). These derivatives were not further purified or chemically characterized. In the text, they are referred to as RCAM-unnitrated, RCAM-mononitrated, RCAM-dinitrated, and RCAM-iodinated BPTI. The assignments used in this manuscript depend on the determination of the modified tyrosine residues reported in the earlier studies of BPTI nitration (Meloun et al., 1968b) and iodination (Sherman and Kassell, 1968).

Protein samples were preexchanged by exposure to pH 12 in D_2O for 2 h, as described earlier (Snyder et al., 1975). Peptides and buffer components were preexchanged by repeated lyophilization from D_2O. A combination buffer was used which included 100 mM KCl, 0.5 mM DSS, 15 mM acetate, 15 mM tris(hydroxymethyl)aminomethane, 15 mM aspartate, and 15 mM phosphate in D_2O. These components contain five groups which titrate in the range pH 5–13, with pK values of 4.8 (acetate), 7.2 (dipotassium phosphate), 8.1 (Tris), 9.8 (aspartate amino group), and 12.4 (tripotassium phosphate). Derivatives with cystine 14–38 intact were studied at a concentration of 2.5 mM. RCAM derivatives were less soluble and did not completely dissolve when attempting to prepare 1.5 mM solutions. Saturated solutions probably were 0.5–1.0 mM in protein, sufficient for accumulating one spectrum in approximately 40 min. Samples of amino acid standard compounds were 4 mM in concentration. Native, RCAM-unnitrated, and the completely reduced and carboxamidomethylated derivative without the preexchange treatment were examined at a pH of 3.40 in a buffer of 15 mM acetate and 0.5 mM DSS. Protein concentrations were 1.6 mM. These acidic low-salt conditions were used since the derivative with all cystines modified was very insoluble in the combination buffer described above at pH 8.6.

The pH's were adjusted with DCl and KOD. The 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 units. The pH's of samples were measured immediately before and after accumulation of spectra. The two values differed by 0–0.02 pH unit in samples studied below pH 8, 0–0.05 pH unit between pH 8 and 10.5, and 0–0.10 pH unit at higher pH's. pH's measured before spectra accumulation were used in figures and titration fits.

^{1}H NMR spectra at 250 MHz were obtained on the MPC-HF 250 MHz super-conducting spectrometer (Dadok et al., 1970). Time averaging 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 300–1200 rapid scans of the spectrum were digitized and accumulated (1500 Hz sweep width, 1.6 s/scan) and then correlated with a calculated reference line with a line width of 0.5 Hz. Ambient temperature was 28 °C. All chemical shifts are expressed relative to a DSS internal standard.

Results

In an earlier manuscript (Snyder et al., 1975), the complete assignments of tyrosine and nitrotyrosine ring resonances in the spectra of unreduced BPTI derivatives were presented together with descriptions of the spectra and titration behavior of model tyrosine and nitrotyrosine compounds and an evaluation of the relative merits of different tyrosine assignment techniques. Although the assignments of resonances in RCAM derivatives to be presented here are made independently of the unreduced BPTI assignments, some familiarity with the techniques evaluated earlier will be assumed below. Protein samples are preexchanged by exposure to high pH in D_2O (see Experimental Section for details) to remove resonances associated with exchangeable NH's which are buried in the interior of BPTI at neutral pH. The resulting aromatic portion of the spectrum (δ_{DSS} = 8.4 to 6.0 ppm) then consists only of resonances associated with the 36 aromatic ring protons. Resonances associated with the 16 tyrosine protons of the four tyrosines of BPTI are distinguished from those associated with the 20 phenylalanine protons of the four phenylalanines on the basis of the titratability of the former. As was true for unreduced BPTI derivatives, RCAM-BPTI derivatives are soluble at concentrations near 1 mM over the pH range 5–13, thereby facilitating titrations. After tyrosine resonances are located, they are assigned to specific residues by selective tyrosine chemical modifications. This is possible since each of BPTI's tyrosines has a unique susceptibility to chemical modification, enabling preparation of a series of four derivatives with four different combinations of tyrosine modifications. These are (1) the unnitrated inhibitor containing unmodified tyrosines 10, 21, 35, and 23, (2) mononitrated BPTI in which tyrosine 10 is nitrated, (3) dinitrated BPTI in which both tyrosines 10 and 21 are nitrated, and (4) iodinated BPTI, an unpurified mixture of derivatives in which tyrosines 10, 21, and 35 are mono- or di-iodinated but in which tyrosine 23 remains unchanged. Following assignment of tyrosine protons to specific residues, further assignments to positions within the ring are

possible. For those tyrosines which appear as two resonances of area two, the resonances are assigned to the 3,5 ortho and 2,6 meta protons on the basis of the relative changes in their chemical shift on titration. The resonance showing the greater change is assigned to the ortho protons.

pH Titration of Tyrosine 35 Resonances in Unreduced Derivatives. The pH titrations of unreduced native, mononitrated, and dinitrated BPTI have been repeated. The additional data obtained support the previous assignments (Snyder et al., 1975), lead to more accurate titration curves, and permit a description of the titration behavior of the three upfield tyrosine 35 resonances not possible from the earlier data.

The results are summarized by closed dots in Figure 4. Revised values for the pK's of tyrosines 10 and 21 are 9.9 and 10.6, respectively. The previously reported values were 10.4 and 11.0. The new values are considered more reliable since they are based on chemical shifts measured at more pH's. Revised values for the pK's of nitrotyrosines 10 and 21 and tyrosines 23 and 35 are 6.5, 6.4, 11.6, and 11.0, respectively, agreeing with the previous values of 6.6, 6.4, 11.7, and 11.1.

As was shown earlier, the spectrum of tyrosine 35 appears as four resolved doublets of area one at pH 5 in dinitrated BPTI. Each resonance corresponds to a particular proton and environment in the immobile ring. Their chemical shifts are summarized in Figure 4D. Resonances A and C previously were shown to be associated with adjacent protons by decoupling experiments. Resonances B and C have been shown to be directly opposite each other by their coalescence into a single resonance at higher temperatures (G. H. Snyder and B. D. Sykes, unpublished results). At 60 °C, rotation of the tyrosine ring about the C_β–C_γ bond is sufficiently rapid to give a single fast-exchange averaged environment for these protons. These results yield the ring positions indicated in Figure 4D. Resonances B and C overlap at pH 11.15, and resonances D and C overlap at pH 12.15. In both cases, the broad nature of the resonance of area two at the point of overlap supports the ring position assignments. If proton C were adjacent to B or D, which is not the case, then superposition of the strongly coupled resonances would yield a sharp singlet having AA′ character. This is not observed. Finally, the A–D pair of protons cannot be assigned to the ortho or meta pair of positions by comparing their changes in chemical shift on titration to the changes occurring in the B–C pair. Resonance A titrates further than resonance C associated with the adjacent proton, but resonance D titrates less than resonance B associated with its adjacent proton. Thus in tyrosine 35, one meta proton resonance titrates further than the corresponding ortho proton resonance.

The titration behavior of the tyrosine 35 resonances B, C, and D is seen most clearly in the left-hand side of Figure 1 which illustrates the titration of dinitrated BPTI from pH 8.60 to 12.15. The titrating tyrosine 35 resonances overlap two stationary nitrotyrosine 21 resonances of area one each. The right-hand side of Figure 1 illustrates the titration of mononitrated BPTI in the same pH interval. Here the nitrotyrosine 21 resonances are replaced by titrating tyrosine 21 resonances of total area equal to four. Tyrosine 35 resonances which are obscured in dinitrated BPTI frequently are resolved in the mononitrated species, and vice versa. Spectra of native BPTI in this region were essentially identical with those of mononitrated BPTI with the exception of extra intensity associated with a tyrosine 10 resonance of area two. These regions of extra intensity in native BPTI spectra are indicated by dotted lines superimposed on the mononitrated inhibitor spectra.

Analysis of Cystines Present in RCAM Derivatives. Although no chemical analyses were performed to determine the number and positions of disulfides in the reduced and carboxamidomethylated derivatives, reasonable assumptions can be made about the principal component of each derivative studied here. The RCAM-unnitrated inhibitor and the completely reduced and carboxamidomethylated derivative were

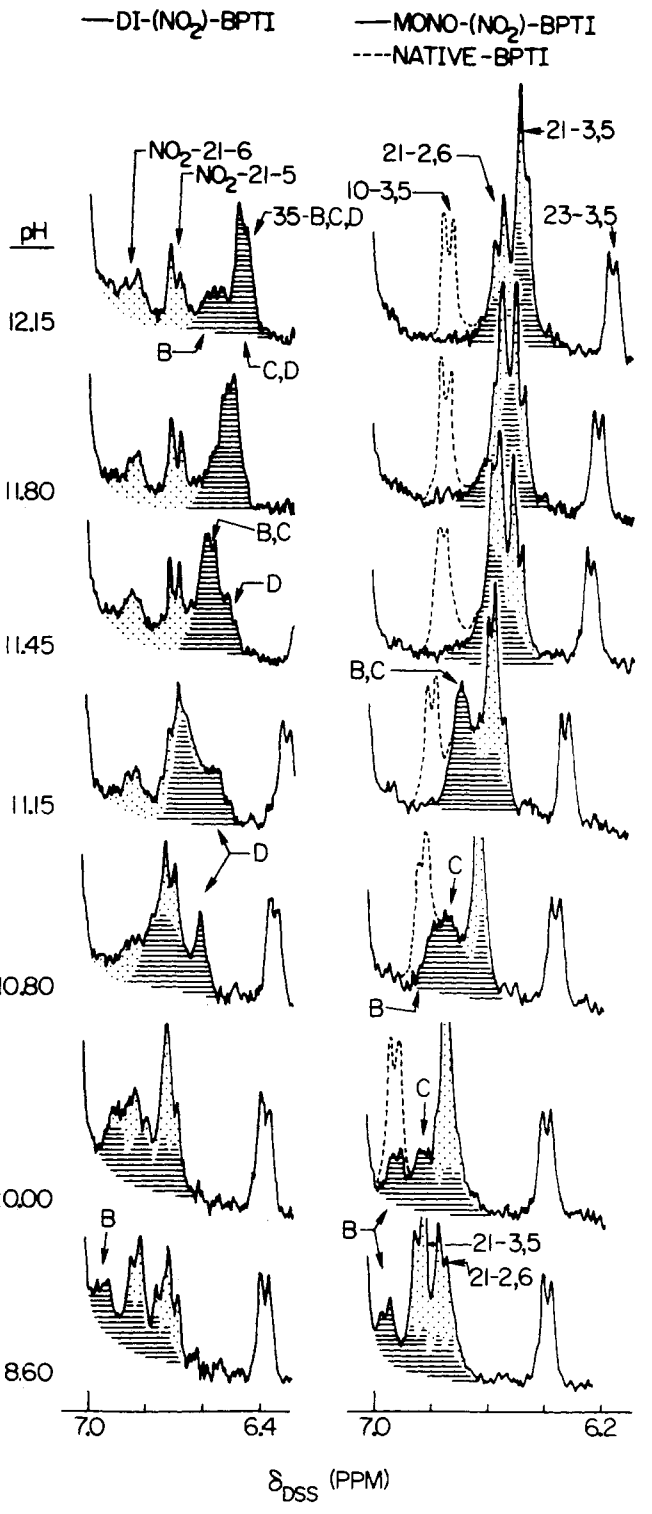

FIGURE 1: Upfield portion (δ_{DSS} = 7.1 to 6.1) of the aromatic region of the 250-MHz NMR spectra of preexchanged mononitrated and dinitrated BPTI: [protein] $\simeq 2 \times 10^{-3}$ M in combination buffer of 15 mM acetate, 15 mM Tris, 15 mM potassium phosphate, and 15 mM aspartate, 0.1 M KCl, 0.1 mM DSS, D_2O, pH as indicated; $\simeq$1000 scans using correlation spectroscopy method. Dotted lines are taken from spectra of preexchanged native BPTI in regions where there is extra intensity not present in spectra of mononitrated BPTI at the same pH's.

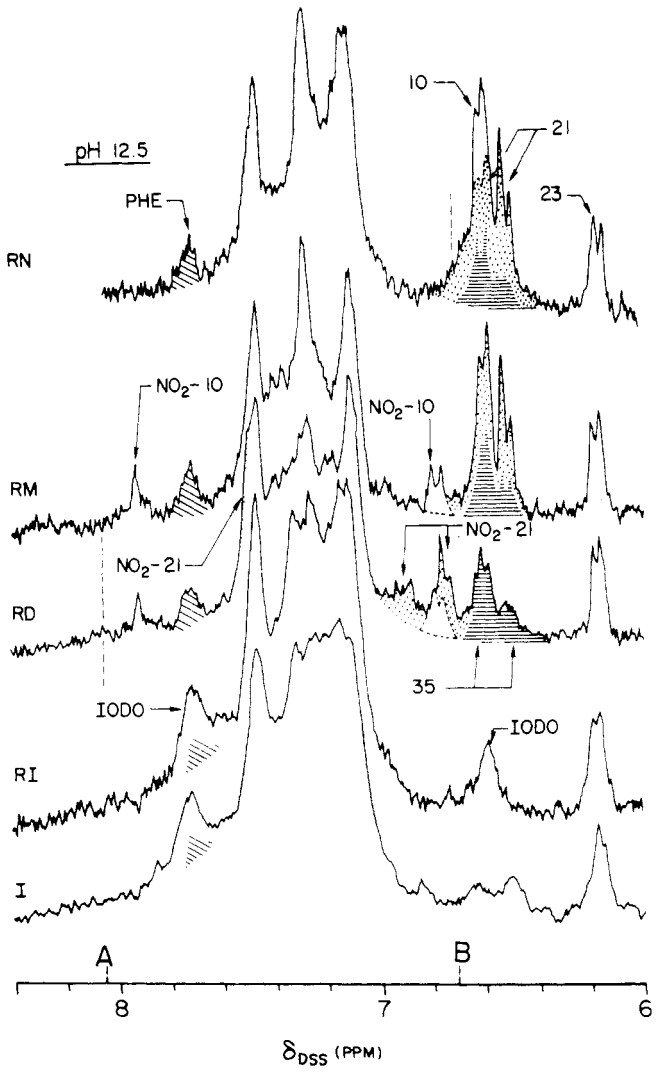

FIGURE 2: Aromatic region (δ_{DSS} = 8.4 to 6.0 ppm) of the 250-MHz NMR spectrum of preexchanged BPTI derivatives; RN = RCAM (unnitrated), RM = RCAM (mononitrated), RD = RCAM (dinitrated), RI = RCAM (iodinated), I = iodinated (see Experimental Section for details); [protein] < 1.3×10^{-3} M in combination buffer of 15 mM acetate, 15 mM Tris, 15 mM potassium phosphate, and 15 mM aspartate, 0.1 M KCl, 0.1 mM DSS, D_2O, pH 12.50; ≃1000 scans using correlation spectroscopy method. A indicates the chemical shift of the 2 proton of nitrotyrosine 10 in both 2 mM mononitrated and 2 mM dinitrated BPTI in the same buffer at the same pH. B indicates 3,5 protons of tyrosine 10 in 4 mM native BPTI.

prepared according to previous studies (Liu and Meienhofer, 1968) in which quantitative modification of cystine 14–38 or all three cystines was observed. Hence, these two derivatives produced here are assumed to have the same composition as the quantitatively analyzed products. The lack of some native BPTI resonances in spectra of RCAM-unnitrated BPTI supports the assumption that the literature preparation of RCAM-unnitrated inhibitor was reproduced in these studies. For example, position B in Figure 2 corresponds to the chemical shift of a tyrosine 10 doublet of area two in the pH 12.6 spectrum of native BPTI. Since there is no corresponding resonance intensity in the RCAM-unnitrated inhibitor spectra, this derivative must lack unmodified native BPTI. Following the disulfide modification treatment on mononitrated or dinitrated BPTI, at least one cystine is modified. The lack of unreduced starting proteins in the final product is shown in spectra of the RCAM products in Figure 2 by the lack of resonances occurring uniquely in spectra of the starting proteins. For example, position A corresponds to the chemical shift of the resolved H-2 singlet of nitrotyrosine 10 in the pH 12.5 spectra of unreduced mononitrated and dinitrated BPTI. There is no corresponding intensity within the limits of signal to noise in spectra of the RCAM-mononitrated and RCAM-dinitrated BPTI products, indicating that following the cystine modification treatment there is no unreduced mononitrated or dinitrated starting protein remaining. Finally, the same batch of iodinated BPTI studied earlier was used in the preparation of the RCAM-iodinated derivative. Following the cystine modification treatment, the RCAM-iodinated product derivative may contain some unreduced iodinated-BPTI, but there is some cystine modification as indicated by the resonance at 6.6 ppm in the spectrum of RCAM-iodinated BPTI in Figure 2 which is not present in the spectrum of the unreduced iodinated inhibitor. RCAM-mononitrated, RCAM-dinitrated, and RCAM-iodinated derivatives do not have all the cystines modified, since they contain an upfield tyrosine resonance at 6.38 ppm at pH 5.0 which is not observed in the pH 3.4 spectrum of the derivative with complete cystine modification. Thus, these three RCAM derivatives must contain either one or two modified cystines. If two cystines were modified, at least one of them would have to be the 5–55 or 30–51 disulfide in the base of the pear-shaped BPTI molecule near tyrosine 23. As will be seen below, tyrosine 23 has the same behavior and hence identical environment in these derivatives as in RCAM-unnitrated BPTI. Thus, it is most likely that RCAM-nitrated and RCAM-iodinated derivatives have intact 5–55 and 30–51 cystines.

Assignment of Tyrosine Resonances to Particular Residues in RCAM Derivatives at pH 12.5. The assignment of tyrosine resonances to specific residues in RCAM derivatives was performed in a manner analogous to the earlier assignments for unreduced derivatives (Snyder et al., 1975). Instead of presenting a complete detailed proof of the RCAM-derivative assignments, only selected portions of the results will be described below. The RCAM assignments are facilitated by the same qualitative characteristics found earlier in unreduced BPTI. As before, derivatives are soluble at concentrations near 1 mM at pH's between 5 and 13. The RCAM-inhibitor molecule maintains its overall structural integrity in the same pH interval, permitting smooth titration curves for tyrosines and nitrotyrosines. Also, resonances associated with any given tyrosine appear to behave independently of chemical modifications or titrations at other tyrosine rings. Finally, RCAM-BPTI is sufficiently structured to give each of its tyrosines different environments and behavior, enabling the resolution of resonances associated with different tyrosines.

The top spectrum of Figure 2 consists of the downfield portion of the pH 12.5 spectrum of RCAM-unnitrated BPTI. Because this derivative was preexchanged, the spectrum consists only of resonances associated with the 36 aromatic ring protons. The downfield resonance at 7.7 ppm is attributed to phenylalanine since it is not titratable with pH. In contrast, all of the resonances upfield of 7.0 ppm shift downfield on lowering the pH from 12.5 to 5.0. They therefore correspond to tyrosine protons. Although the spectrum was not accumulated under conditions ensuring complete relaxation of resonances, all aromatic resonances have similar T_1's of 1.4 ± 0.2 s at 270 MHz (G. H. Snyder and B. D. Sykes, unpublished results). Thus, comparisons of relative areas permit analysis of relative numbers of associated aromatic ring protons.

Comparing the spectrum of RCAM-unnitrated inhibitor in Figure 2 with the spectrum of the RCAM-mononitrated

inhibitor directly below it, nitration causes the disappearance of intensity of area two at 6.58 ppm and the new appearance of two resolved resonances of area one, a singlet at 7.88 ppm and a doublet at 6.77 ppm. Therefore, these resonances are assigned to tyrosine 10 and nitrotyrosine 10, respectively. Similarly, a comparison of the spectra of RCAM-mononitrated and RCAM-dinitrated derivatives reveals the loss of both tyrosine 21 doublets with a total area of four centered at 6.53 ppm and the new appearance of two nitrotyrosine 21 doublets of area one at 6.85 and 6.70 ppm. Since the upfield tyrosine doublet of area two at 6.13 ppm is present in the spectrum of RCAM-iodinated inhibitor, as well as the spectra of the three previously discussed RCAM derivatives, it is assigned to the chemically inert tyrosine 23. pH titration of the RCAM-iodinated derivative reveals that the resonance in its spectrum at 6.6 ppm is titratable but has a different pK from the tyrosine 23 resonance. It therefore must be attributed to iodotyrosine protons. Hence, the tyrosine resonances at 6.6 ppm in the RCAM-dinitrated derivative are not present in RCAM-iodinated BPTI and must be associated with tyrosine 35.

pH Titration of RCAM Derivatives. The left-hand side of Figure 3 illustrates the titration of RCAM-dinitrated BPTI from pH 5 to 12, considering only the aromatic resonances appearing upfield of 7.1 ppm. The pH 5 spectrum consists of a broad tyrosine 35 resonance labeled F and a tyrosine 23 resonance of area two. After raising the pH to 8.60, these tyrosine resonances remain stationary but are overlapped by nitrotyrosine resonances titrating upfield into the region shown. These latter resonances consist of two resonances of area one associated with nitrotyrosine 21 and one resonance of area one associated with nitrotyrosine 10. On raising the pH from 8.60 to 10.80 and finally to 12.15, the nitrotyrosine resonances remain stationary but tyrosine resonances titrate upfield. A new tyrosine 35 resonance labeled E titrates upfield at pH 10.80 into the region shown. At pH 12.15, the total tyrosine 35 area upfield of 7.1 ppm corresponds to three protons, E, F, and G. It has not been possible to determine the location of resonance G at lower pH's. It may overlap either resonance E or F. The location of resonance H associated with the fourth remaining tyrosine 35 proton is presumed to be located somewhere in the region of overlapping tyrosine and phenylalanine resonances between 7.0 and 7.4 ppm. Because the E,G resonance at pH 12.15 lacks AA′ singlet character, protons E and G are located on opposite sides of the ring as indicated in Figure 4D.

In spectra of RCAM-mononitrated BPTI in the center of Figure 3, the nitrotyrosine 21 resonances are replaced by tyrosine 21 resonances of total area corresponding to four protons. Tyrosine 21 appears as two doublets of area two at pH's 8.60 and 12.15. At pH 10.80 these resonances superimpose, producing a singlet of area four with AA′ character. In the right-hand side, spectra of RCAM-unnitrated BPTI contain a tyrosine 10 resonance of area two in place of the previous nitrotyrosine 10 resonance.

As before, changes which occur in the region between 7.4 and 7.0 ppm were detected by direct superposition of spectra whose vertical scales were adjusted to give equal intensities for the resolved upfield tyrosine 23 doublet. This region corresponds to overlapping phenylalanine and tyrosine resonances. Titratable changes were assigned to tyrosine resonances and cross-checked by matching their areas and pK's with tyrosine resonances located outside of this region. Resonances have been located for all the aromatic protons of tyrosines 10, 21, and 23 and nitrotyrosines 10 and 21. Resonances associated with three of the four tyrosine 35 protons have been located at pH 12.15, with two protons accounted for at lower pH's.

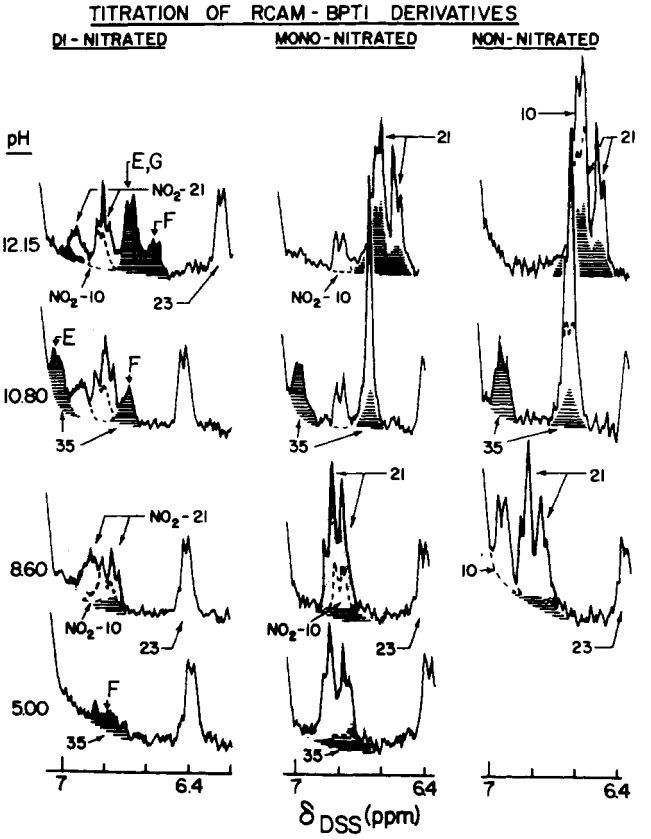

FIGURE 3: Upfield portion (δ_{DSS} = 7.1 to 6.2 ppm) of the aromatic region of the 250-MHz NMR spectrum of preexchanged RCAM-dinitrated, RCAM-mononitrated, and RCAM-unnitrated BPTI: [protein] $< 1.3 \times 10^{-3}$ M in combination buffer of 15 mM acetate, 15 mM Tris, 15 mM potassium phosphate, and 15 mM aspartate, 0.1 M KCl, 0.1 mM DSS, D_2O, pH as indicated; $\simeq$900 scans using correlation spectroscopy method.

The positions of nitrotyrosine and tyrosine resonances in BPTI derivatives before and after reduction and carboxamidomethylation of cystine 14–38 are summarized in Figure 4. Resonances of derivatives with the cystine intact are indicated by closed dots. Resonances of derivatives in which the disulfide has been modified are indicated by open circles. Overlapping resonances associated with the same ring are indicated by vertical lines drawn through the dots or circles, as seen in graphs for tyrosines 21 and 35 and nitrotyrosine 21. When the chemical shift of a particular resonance at a particular pH was determined in derivatives having the same cystine 14–38 status, but different tyrosine modifications, the values were identical within experimental error and were averaged to give a single point on the graph. These averaged chemical shifts were fit using nonlinear least-square method to a one proton titration curve (eq 1)

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

with δ_{HA}, δ_{A^-}, and K_a as parameters of the fit. For tyrosines 21 and 23 and nitrotyrosine 21, the titration behavior was identical in RCAM and unreduced derivatives. For these rings, the RCAM and unreduced BPTI data were combined and refit to give the single curves in Figures 4A and 4B. The results are summarized in Table I.

Further Assignment to Positions within Individual Tyrosine and Nitrotyrosine Rings. The assignments of resonances to ortho or meta positions within the rings of tyrosines 21 and 23 and nitrotyrosine 21 are identical for the RCAM and unreduced derivatives. They were discussed in detail previously for

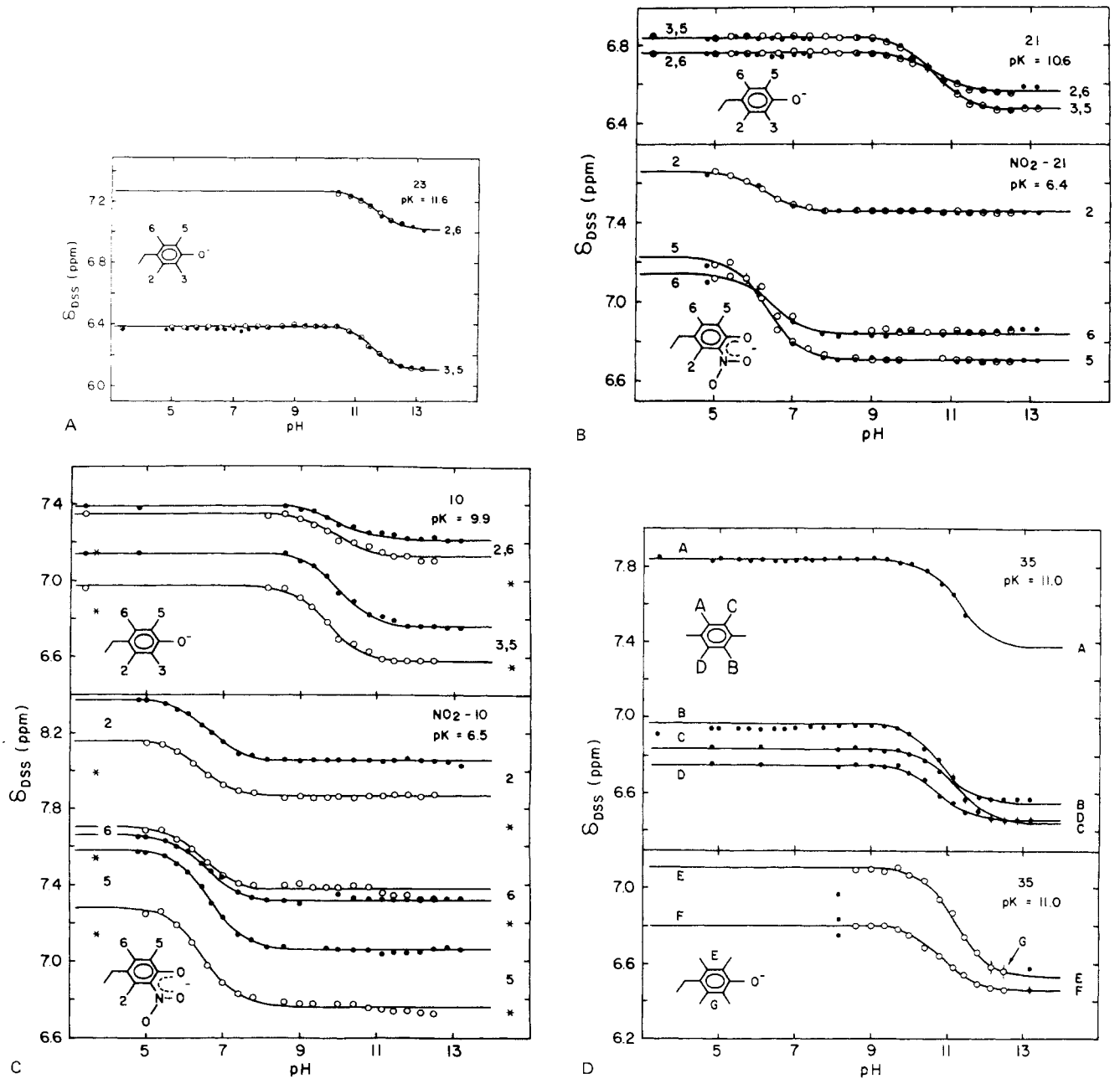

FIGURE 4: (A–D) Chemical shifts of all tyrosine and nitrotyrosine resonances in the aromatic region (δ_{DSS} = 9.0 to 8.0) of the 250-MHz NMR spectrum of preexchanged unnitrated, mononitrated, and dinitrated BPTI (before and after reduction and carboxamidomethylation of cystine 14–38) as a function of pH; [protein] $\simeq 2 \times 10^{-3}$ M in combination buffer of 15 mM acetate, 15 mM Tris, 15 mM potassium phosphate, and 15 mM aspartate, 0.1 M KCl, 0.1 mM DSS, 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's are the calculated nonlinear least-squares fits to single proton titration curves (see Results for details). Curves are the computed fits. Symbols indicating the status of cystine 14–38 are: (●) intact disulfide bridge; (○) bridge reduced and carboxamidomethylated. Points representing the overlap of two resonances are indicated by vertical lines. Asterisks represent chemical shifts observed in *N*-acetyl-L-tyrosinamide and *N*-acetyl-3-nitro-L-tyrosine ethyl ester under similar conditions. Peptide resonances appear in the same order as observed in the tyrosine 10 and nitrotyrosine 10 resonances.

the unreduced derivatives (Snyder et al., 1975). The determination that resonances of the unmodified and nitrated tyrosine 21 ring are crossing rather than noncrossing during titration was based on the observation that the crossing treatment gives closer agreement in pK's. The resonances of unmodified and nitrated tyrosine 10 in RCAM derivatives were assigned to ring positions using the techniques described earlier. For example, the H-2 proton of the nitrotyrosine ring is distinguished on the basis of its singlet character. The nitrotyrosine doublet which titrates the furthest on changing pH is assigned to the H-6 ortho proton, with the remaining doublet assigned to the H-5 meta proton. The resonances of tyrosine 35 will not be assigned to ortho or meta positions. However, some information can be obtained about the relative positions of their associated protons by considerations of intensity patterns, decoupling experiments, and temperature series. These considerations were discussed above and are summarized in Figure 4D.

Assignments in the Completely Reduced and Carboxamidomethylated Derivative. Finally, the aromatic region of the pH 3.4 spectrum of the derivative with all three disulfides reduced and carboxamidomethylated is presented in the center of Figure 5. The regions above and below 7.0 ppm have relative areas of 8.0 and 28.0, respectively, such that the upfield mul-

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

Resonance			Parameters					
Ring	Proton position	Comment[c]	δ_{HA}[a,d]	δ_{A^-}[a,d]	pK[b,d]	δ_{HA}[a,d]	δ_{A^-}[a,d]	pK[b,d]
			N, M, D, RN, RM, RD					
21	2,6	C	6.76	6.57	10.5			
	3,5	C	6.84	6.48	10.6			
23	2,6		7.27	7.02	11.7			
	3,5		6.39	6.10	11.6			
NO_2-21	2		7.66	7.46	6.3			
	6	C	7.14	6.84	6.5			
	5	C	7.22	6.71	6.4			
21	Downfield	NC	6.85	6.57	10.3			
	Upfield	NC	6.76	6.47	10.8			
NO_2-21	Downfield	NC	7.21	6.84	6.3			
	Upfield	NC	7.15	6.70	6.5			
			N, M, D			RN, RM, RD		
10	2,6		7.39	7.22	10.0	7.35	7.13	9.9
	3,5		7.14	6.76	10.0	6.97	6.58	9.7
35	A		7.84	7.38	11.3			
	B		6.97	6.55	10.8			
	C		6.83	6.44	11.2			
	D		6.75	6.46	10.8			
	E					7.11	6.53	11.2
	F					6.80	6.46	10.8
NO_2-10	2		8.39	8.08	6.6	8.17	7.87	6.4
	6		7.68	7.34	6.6	7.70	7.37	6.5
	5		7.60	7.08	6.7	7.28	6.76	6.5

[a] Chemical shift (±0.02 ppm) from DSS. [b] ±0.1 pK unit. [c] C, resonances chosen as crossing, NC, resonances chosen as noncrossing; N, native, M, mononitrated, D, dinitrated, RN-RCAM (unnitrated), RM-RCAM (mononitrated), RD-RCAM (dinitrated). [d] Averaged data from several derivatives.

tiplet exactly aligned with the standard tyrosine ortho position tentatively is attributed to the eight ortho protons of the derivative's four tyrosine rings.

Discussion

Effects of Cystine 14-38 Modification on Tyrosine 10 and Nitrotyrosine 10. The titration of tyrosine 10 and nitrotyrosine 10 resonances is summarized in Figure 4C. Reduction and carboxamidomethylation of cystine 14-38 produce similar chemical shift effects in both rings. For example, resonances in general shift upfield toward model peptide positions, suggesting partial relaxation of the local environmental influences which caused the original nonstandard downfield chemical shifts. In both rings, the ortho protons experience a greater effect than the meta protons. Finally, changes in chemical shift at low pH are approximately equal to those at high pH, indicating that the effects are independent of the presence or absence of charge at the tyrosine or nitrotyrosine ring.

The second inflection near pH 11 in the titration curves of the H-6 and H-5 resonances in RCAM nitrated derivatives may be due to interaction with nearby lysine 41. Unreduced nitrated derivatives do not appear to show the same effect. The pK's do not change as a result of cystine modification. Tyrosine 10 has a pK of 9.9, already equal to the model tyrosine peptide value of 10.0. In contrast, nitrotyrosine 10 has a pK of 6.5, different from the model nitrotyrosine value of 7.1. There may be separate environmental influences contributing to this ring's chemical shifts and pK's, or its chemical shifts may be more sensitive to small environmental changes than its pK.

Effects of Cystine 14-38 Modification on Tyrosine 35. The

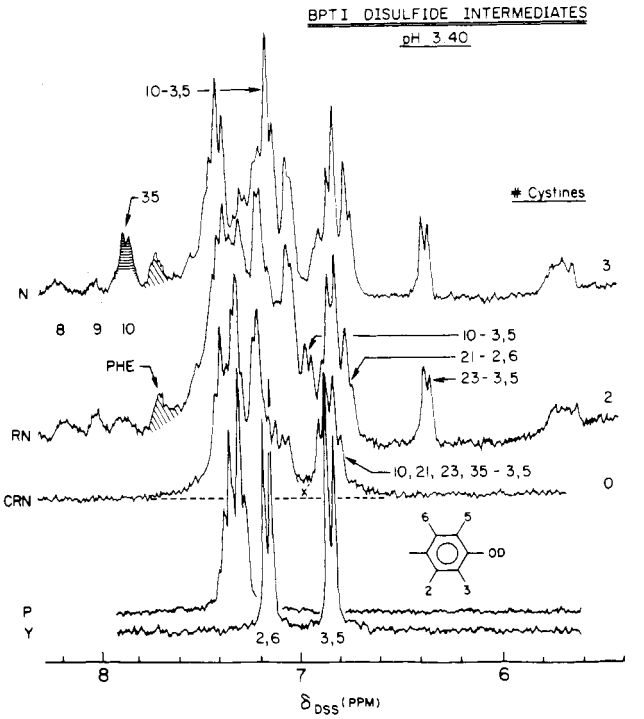

FIGURE 5: Aromatic region (δ_{DSS} = 8.3 to 5.4) of the 250-MHz NMR spectrum of BPTI derivatives and amino acid standards; N = native BPTI, RN = RCAM-unnitrated BPTI, CRN = native BPTI completely reduced and carboxamidomethylated. P = *N*-acetyl-L-phenylalaninamide, Y = *N*-acetyl-L-tyrosinamide; [protein] = 1.6×10^{-3} M, [peptide] = 10×10^{-3} M in 15 mM acetate buffer, 0.1 M KCl, 0.1 mM DSS, D_2O, pH 3.40, samples aged 12 h prior to accumulating data; ≃500 scans using correlation spectroscopy method.

titration of tyrosine 35 resonances is summarized in Figure 4D. Positions of the upfield resonances in the unreduced derivatives at pH's 8.15 and 13.20 have been copied onto the graph presenting the shifts in RCAM-BPTI to facilitate comparisons. As was true for tyrosine 10 and nitrotyrosine 10, the cystine 14–38 modification introduced changes in chemical shifts without altering the pK. No attempt will be made to correlate resonances A, B, C, and D before cystine modification with resonances E, F, or G in the RCAM derivatives.

Three features of the behavior of tyrosine 35 are conserved following the cystine modification. First, at pH 12.5 resonances associated with three protons appear between 6.46 and 6.56 ppm. At least one of these must be a meta resonance shifted at least 0.45 ppm upfield of the model peptide shift of 6.99 ppm, indicating conservation of a factor causing upfield shifts in at least one meta proton's environment.

Secondly, in both unreduced and RCAM derivatives there are indications that the titration of tyrosine 35 may be accompanied by changes in its environment. In unreduced derivatives resonances A, B, C, and D titrate 0.46, 0.42, 0.39, and 0.29 ppm, respectively. Thus, inductive effects resulting from deprotonation are not symmetrical on the two sides of the ring. Either the inductive effects are influenced by different amounts by the environments on opposite sides of the immobile ring or a change in environments accompanies titration of tyrosine 35. In RCAM derivatives resonance E shifts by 0.58 ppm during titration. The largest changes observed in the resonances of the other three RCAM-BPTI tyrosines are 0.39, 0.36, and 0.29 ppm for the ortho resonance of tyrosines 10, 21, and 23, respectively, compared with 0.29 ppm in the ortho resonance of N-acetyl-L-tyrosinamide. The unusually large value of 0.58 ppm for resonance E therefore suggests that inductive effects resulting from deprotonation are insufficient to explain the observed change. An environmental change which adds a source of an upfield shift or removes a source of a downfield shift when raising the pH would provide the additional contribution necessary to explain the observed shifts.

Finally, environmental barriers to rotation are conserved. The tyrosine ring rotates less than 1600 times/s at 28 °C and pH's between 11 and 13 in both unreduced and RCAM derivatives. In RCAM-BPTI, the only clearly resolved tyrosine 35 resonance of area one is resonance F at pH's above 10.8, as seen in Figure 3. The area one resonance corresponding to the proton directly opposite proton F must appear in the region between 7.4 and 6.4 ppm, no further than 1.0 ppm from resonance F. At pH's above 10.8, rotation about the C_β–C_γ bond is not fast enough to give the pair of protons the same averaged environment. Application of the slow-exchange relationship ($\Delta\omega = 1600$ rad s$^{-1} > 1/\tau$ s^{-1}) then places an upper bound of 1600 times/s on the rate ($1/\tau$) of rotation of tyrosine 35. In unreduced BPTI, the 0.1-ppm separation between resonances B and C results in the more stringent upper bound of 160 times/s.

Comparison of NMR Results with Other Studies on RCAM Derivatives. The NMR studies of RCAM-BPTI derivatives suggest that their structure is only slightly changed from that existing in the unreduced molecules. The alkaline titration curves for tyrosine 21 and 23 resonances are exactly superimposable at 25 °C in 0.1 M KCl, as are the acidic titration curves for nitrotyrosine 21 resonances. Nitrotyrosine 10 and tyrosines 10 and 35 show some changes in chemical shift, but nitrotyrosine 10 and tyrosine 35 retain their nonstandard pK's and the tyrosine 35 ring retains its immobility. The distances from the centers of the rings of tyrosines 35, 10, 21, and 23 to the center of the cystine 14–38 disulfide in native BPTI are 7.6, 9.3, 20.0, and 24.6 Å, respectively, calculated on the basis of the known crystal structure (Deisenhofer and Steigemann, 1974). Thus, changes occur in tyrosine environments which are at distances less than 10 Å from cystine 14–38 but not at those more than 20 Å away. In addition, the cystine modification does not alter the nonstandard chemical shifts of the aliphatic resonances at 5.7 ppm or the phenylalanine resonance at 7.7 ppm. Moreover, as seen in Figure 5, the chemical shifts and exchange rates (at pH 3.40 after 12 h in D_2O) of at least nine NH resonances resolved downfield from 7.7 ppm are similarly unchanged.

These results are consistent with earlier laser Raman (Brunner et al., 1974) and CD/ORD (Vincent et al., 1971) spectroscopic studies showing few differences at physiological conditions when comparing RCAM-unnitrated and native BPTI. The aggregate alkaline isomerization curves measured as $\Delta[m]_{230nm}$ between pH 7.5 and 11.5 were exactly superimposable for RCAM-unnitrated and native BPTI at 25 °C in 0.1 M NaCl. Reciprocal changes in tyrosine residues would have gone undetected. Other results have demonstrated that RCAM-unnitrated BPTI is able to bind to trypsin and inhibit its activity (Vincent and Lazdunski, 1972). The NMR results provide more detailed information about the location of structural changes, since resonances associated with residues in four different locations in the molecule can be individually assigned and monitored.

Implications for Protein Folding. The alignment of spectra of three disulfide derivatives in Figure 5 demonstrates that sequential formation of disulfides is accompanied by sequential acquisition of native structure. In the spectrum of the derivative with no disulfides, the ortho resonances form a quartet-like multiplet instead of aligning perfectly to form a doublet of area eight. Thus, the four tyrosines have nonequivalent environments, suggesting that this derivative is not a completely random coil. Without disulfides, the polypeptide chain is however flexible enough such that all NH's exchange with D_2O solvent after 12 h at pH 3.40. During oxidation of completely reduced BPTI, both the 5–55 and 30–51 disulfides must be intact before a cystine 14–38 linkage is formed (Creighton, 1975a,b). At the point in time where these first two disulfides are formed, some of the native structural features are present. This is particularly true near the base of the pear-shaped inhibitor molecule where the 5–55 and 30–51 disulfides are located, as evidenced by the behavior of the nearby tyrosines 21 and 23. Additional native-like environments exist for the phenylalanine and α-CH protons whose resonances appear at 7.7 and 5.7 ppm, respectively. Moreover, of the 10 NH's which previously were observed not to exchange with D_2O solvent in native BPTI after 2 days at pH 7 (Karplus et al., 1973), NH's "2"–"10" have their native buried environments with no apparent changes in chemical shifts or exchange rates. Spectra were not accumulated sufficiently downfield to observe the behavior of the NH "1" resonance at 10.8 ppm. Finally, the 3,5 ortho resonance of tyrosine 10 progressively acquires its nonstandard downfield shift as more disulfides are formed in the derivatives included in Figure 5. With no disulfides present it has a chemical shift near 6.8 ppm, essentially identical with the exposed ortho resonances in the peptide standard. In RCAM-unnitrated BPTI, it has shifted downfield to 6.9 ppm, having moved part of the way toward its final native position of 7.2 ppm.

Acknowledgments

The authors thank Professor A. Bothner-By for helpful discussions and financial support and Professor M. Karplus

for encouraging the study of reduced BPTI. The authors also are indebted to T. D. Marinetti for providing mono- and dinitrated inhibitor derivatives, to K. Seamon for technical assistance, and to Farbenfabriken Bayer AG for a generous gift of trypsin inhibitor (Trasylol). One of us (G.H.S.) especially appreciates the hospitality extended to him by Dr. N. Rowan during numerous trips to Pittsburgh.

References

Arata, Y., Khalifah, R., and Jardetzky, O. (1973), *Ann. N.Y. Acad. Sci. 222,* 230.
Benz, F. W., and Roberts, G. C. K. (1975a), *J. Mol. Biol. 91,* 345.
Benz, F. W., and Roberts, G. C. K. (1975b), *J. Mol. Biol. 91,* 367.
Brunner, H., Holz, M., and Jering, H. (1974), *Eur. J. Biochem. 50,* 129.
Creighton, T. E. (1974a), *J. Mol. Biol. 87,* 579.
Creighton, T. E. (1974b), *J. Mol. Biol. 87,* 603.
Creighton, T. E. (1975a), *J. Mol. Biol. 95,* 167.
Creighton, T. E. (1975b), *J. Mol. Biol. 96,* 767.
Creighton, T. E. (1975c), *J. Mol. Biol. 96,* 777.
Dadok, J., and Sprecher, R. F. (1974), *J. Magn. Reson. 13,* 243.
Dadok, J., Sprecher, R. F., Bothner-By, A. A., and Link, T. (1970), 11th Experimental Conference, Pittsburgh, Pa., Abstracts, Section C-2.
Deisenhofer, J., and Steigemann, W. (1974), in 2nd International Research Conference on Proteinase Inhibitors (Bayer Symposium V), Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E., Ed., Springer-Verlag, New York, Heidelberg, West Berlin.
Karplus, S., Snyder, G. H., and Sykes, B. D. (1973), *Biochemistry 12,* 1323.
Kress, L. F., and Laskowski, M., Sr. (1967), *J. Biol. Chem. 242,* 4925.
Liu, W., and Meienhofer, J. (1968), *Biochem. Biophys. Res. Commun. 31,* 467.
Liu, W., Trzeciak, H., Schussler, H., and Meienhofer, J. (1971), *Biochemistry 10,* 2849.
Meloun, B., Fric, I., and Sorm, F. (1968a), *Collect. Czech. Chem. Commun. 33,* 2299.
Meloun, B., Fric, I., and Sorm, F. (1968b), *Eur. J. Biochem. 4,* 112.
Sherman, M. P., and Kassell, B. (1968), *Biochemistry 7,* 3634.
Snyder, G. H., Rowan, R., III, Karplus, S., and Sykes, B. D. (1975), *Biochemistry 14,* 3765.
Vincent, J. P., Chicheportiche, R., and Lazdunski, M. (1971), *Eur. J. Biochem. 23,* 401.
Vincent, J. P., and Lazdunski, M. (1972), *Biochemistry 11,* 2967.
Westmoreland, D. G., and Matthews, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 914.

Anion-Induced Increases in the Rate of Colchicine Binding to Tubulin†

B. Bhattacharyya and J. Wolff*

ABSTRACT: The rate of binding of colchicine to tubulin is enhanced by certain anions. Among the inorganic anions tested, only sulfate was effective. The organic anions include mostly dicarboxylic acids, among which tartrate was the most effective. This effect occurs only at low concentrations of colchicine ($<0.6 \times 10^{-5}$ M). The rate increase for sulfate and L-(+)-tartrate is ca. 2.5-fold at 1.0 mM and plateaus at a limiting value of ca. 4-fold at 100 mM. The overall dissociation rate of the colchicine from the complex, which includes both the true rate of dissociation and the rate of irreversible denaturation of tubulin, is not influenced by 1.0 mM tartrate. The affinity constants for colchicine determined from the rate constants are 8.7×10^6 and 2.1×10^7 M^{-1} in the absence and the presence of 1.0 mM L-(+)-tartrate. The limiting value is 3.2×10^7 M^{-1}. The affinity constant calculated from steady-state measurements is 3.2×10^6 M^{-1} with or without anions. The binding of other ligands like podophyllotoxin, vinblastine, and 1-anilino-8-naphthalenesulfonate to tubulin is not affected by tartrate. No major conformational changes resulting from anion treatment could be detected by circular dichroism or intrinsic fluorescence. However, the ability of tubulin to polymerize is inhibited by L-(+)-tartrate at concentrations that increase the rate of colchicine binding. We conclude that anions must have a local effect at or near the binding site which enhances the binding rate of colchicine and which may be related to inhibition of polymerization.

Tubulin, the heterodimeric subunit of microtubules, is characterized by its ability to bind colchicine, podophyllotoxin, vinblastine, GTP, and anilinonaphthalenesulfonic acid (Wilson, 1970; Owellen et al., 1972; Olmsted and Borisy, 1973; Jacobs et al., 1974; Bhattacharyya and Wolff, 1975a). With the exception of colchicine, these ligands appear to bind rapidly to tubulin (Taylor, 1965; Wilson, 1970; Owellen et al., 1972). Colchicine binds slowly with a high temperature coefficient and shows equilibration times of 30–90 min, depending on the colchicine concentration. The reason for this slow binding is obscure, although it has been suggested by Ventilla et al. (1972) that colchicine binding is accompanied by a slow conformational change in the tubulin accompanied by a loss in α-helix content.

† From the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received November 14, 1975.*