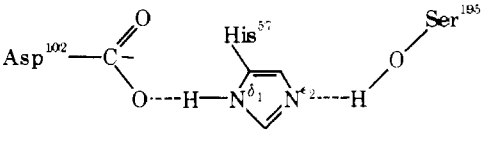
Hydrogen Bonds in Serine Proteinases and Their Complexes with Protein Proteinase Inhibitors. Proton Nuclear Magnetic Resonance Studies†

John L. Markley‡

ABSTRACT: A technique is presented for solvent subtraction in high-resolution proton correlation ^1H nuclear magnetic resonance spectroscopy of proteins in ^1H_2O solution. Single proton peaks of proteins have been resolved in 10 min using 0.6-μmol samples. The technique was used at 250 and 360 MHz to study several low-field, hydrogen-bonded N-H resonances of serine proteinases including the peak previously assigned [Robillard, G., & Shulman, R. G. (1972) *J. Mol. Biol. 71*, 501-511] to the proton hydrogen bonded between active site groups Asp^{102} and His^{57} (chymotrypsinogen numbering system). In porcine trypsin at pH 3.5, the intensity of the N-H peak at 18 ppm downfield from 3-trimethylsilyl-1-propanesulfonic acid sodium salt is 0.6 ± 0.1 proton. The peak is much broader than other N-H peaks in the spectrum, and its width increases sharply with temperature between 0 and 35 °C (where it is no longer detected). These results are consistent with the existence of a conformational equilibrium between two states having lifetimes in the 0.5-2-ms range. In complexes between serine proteinases and protein proteinase inhibitors this conformational equilibrium is abolished, and a number of new hydrogen bonds are formed. Among these is one giving rise to a peak in the 15 to 13 ppm region previously assigned [Robillard, G., & Shulman, R. G. (1974) *J. Mol. Biol. 86*, 519-540] to the above active site proton in the complex between bovine chymotrypsin and bovine pancreatic trypsin inhibitor (Kunitz). This resonance has been studied in nine different proteinase:proteinase inhibitor complexes. In stable complexes the peak has the same width at room temperature as other hydrogen bonded N-H peaks, has an intensity of 1 proton, and has a pH-independent chemical shift which is characteristic of the proteinase and not of the proteinase inhibitor component of the complex. The chemical shift of the peak is identical (13.6 ± 0.1 ppm) in complexes between bovine trypsin or bovine trypsinogen and bovine pancreatic trypsin inhibitor (Kunitz). Similarly, the chemical shift of the peak is identical (14.7 ± 0.1 ppm) in complexes of bovine chymotrypsin A_δ or bovine chymotrypsinogen A with bovine pancreatic trypsin inhibitor (Kunitz). The peak in the latter complex is broad; this indicates that the complex is weak and short-lived. In the complex between porcine trypsin and bovine pancreatic trypsin inhibitor (Kunitz) the peak at 14.3 ppm disappears as the pH is lowered through 3.5. Thus the hydrogen bond is disrupted when the complex undergoes the low pH conformational transition that affects histidine-57. The peak is present at 14.1 ± 0.1 ppm in the complex between porcine anhydrotrypsin and bovine pancreatic trypsin inhibitor (Kunitz); its chemical shift is not significantly different from the analogous peak in spectra of the porcine trypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex. The half-time for deuterium exchange of the N-H proton at 14.3 ppm in the porcine trypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex in ^2H_2O solution is less than 4×10^2 s, which is over two orders of magnitude shorter than the half-time for dissociation of the complex.

Proton nuclear magnetic resonance spectroscopy (^1H NMR)[1] is an attractive tool for studying hydrogen bonding in peptides and proteins. In aqueous solution the appearance of a low-field deuterium-exchangeable peak generally implies the existence of a hydrogen bond whose strength and stability may be inferred from NMR parameters as the temperature or isotopic composition of the solution ($^1H_2O/^2H_2O$) is varied. In small peptides, unambiguous assignments of N-H peaks have been obtained using double resonance techniques, chemical modification, and isotopic substitution (for a recent review, see Wüthrich, 1976). Assignments in proteins are much more difficult because of the larger number of resonances, their greater line width, and the more limited repertoire of assignment techniques. Nevertheless, assignments of N-H peaks in several proteins have been proposed (Glickson et al., 1971; Stellwagen & Shulman, 1973; Patel et al., 1975; Campbell et al., 1975, 1978; Fung et al., 1977). Among these is the assignment by Robillard & Shulman (1972) of a resonance which appears at low temperature to the proton hydrogen bonded between His^{57} and Asp^{102} of the "charge relay system" (eq 1) of serine proteinases (Blow et al., 1969).



(1)

The validity of this experiment was questioned by Hunkapiller et al. (1973) who were unable to reproduce the spectra

† From the Biochemistry Division, Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received April 24, 1978.* Supported by the National Institutes of Health Grants GM 19907 to the Purdue Research Foundation, RR 00711 to the Stanford Magnetic Resonance Laboratory, and RR 00292 to the NMR Facility for Biomedical Studies, Carnegie-Mellon University, and by National Science Foundation Grant GR 23633 to the Stanford Magnetic Resonance Laboratory.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; DSS, 3-trimethylsilyl-1-propanesulfonic acid sodium salt (2,2-dimethyl-2-silapentane-5-sulfonate); BPTI, bovine pancreatic trypsin inhibitor (Kunitz); STI, soybean trypsin inhibitor (Kunitz); BCtg, bovine chymotrypsinogen A; $BCtr_\delta$, bovine chymotrypsin A_δ; BTg, bovine trypsinogen; BTr, bovine trypsin; PTr, porcine trypsin; anhydroPTr, porcine anhydrotrypsin. A colon between two species represents a stable complex, e.g., PTr:BPTI, the complex between porcine trypsin and bovine pancreatic trypsin inhibitor. Notation used follows the "Recommendation for the Presentation of NMR Data for Publication in Chemical Journals" [(1976) *Pure Appl. Chem. 45*, 219]. The chymotrypsinogen numbering system is used for specifying residues of all related serine proteinases.

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of Robillard & Shulman. The original studies (Robillard & Shulman, 1972, 1974a,b) did not explain why the N-H peaks could be observed only at low temperature. The Asp^{102}···His^{57} hydrogen bond is thought to be stable, and hydrogen-bonded His N-H peaks of other proteins are readily resolved at room temperature (Stellwagen & Shulman, 1973; Patel et al., 1975).

I describe here further investigations of serine proteinases and their complexes using rapid-scan, correlation spectroscopy (Dadok & Sprecher, 1974) which permits the resolution of N-H peaks much faster than conventional continuous wave signal averaging. The studies of Robillard & Shulman (1972, 1974a,b) have been replicated in part and have been extended to porcine trypsin and several proteinase-inhibitor complexes. Data for a number of low-field N-H resonances are reported. The requirement of low temperature for the resolution of certain peaks is explained, and some questions concerning the assignments of these peaks are discussed. Solution studies of hydrogen bonds in serine proteinases, their zymogens, and inhibitor complexes are of timely interest as a result of the recent high-resolution X-ray structures of these species (Bode & Huber, 1976; Bode et al., 1976a,b; Fehlhammer et al., 1977; Huber et al., 1974, 1975; Kossiakoff et al., 1977; Sweet et al., 1974).

Experimental Section

Materials. Several proteins were purchased from Worthington Biochemical Corp.: bovine trypsinogen (1× crystallized), bovine trypsin (3× crystallized), bovine chymotrypsinogen A (5× crystallized), bovine chymotrypsin A_δ, bovine chymotrypsin A_α, and soybean trypsin inhibitor (Kunitz). Bovine chymotrypsin A_δ was also made from the zymogen by rapid activation (Garel & Labouesse, 1973). Porcine trypsin was from Enzyme Development Corp. (Novo Industri, A/S, lot no. 5-01-3). The porcine trypsin used was a 9:1 mixture of β- and α-trypsins. No effects attributable to this heterogeneity were detected. Bovine pancreatic trypsin inhibitor (Trasylol, registered trademark of Bayer AG) was a generous gift from Bayer AG. Porcine anhydrotrypsin was a gift from G. H. Weidener and M. Laskowski, Jr. UltraPure potassium chloride was purchased from Alfa Chemical Co. Deuterium oxide (^2H_2O), 99.8% isotopically pure, was purchased from Bio-Rad Labs. Comparable spectra were obtained using either commercial ^2H_2O or ^2H_2O deionized by passage through a Chelex (Bio-Rad Labs.) column.

Preparation of Complexes. Complexes between bovine chymotrypsinogen A and bovine pancreatic trypsin inhibitor (BPTI) and between porcine anhydrotrypsin and BPTI were formed by dissolving the appropriate weights of proteins in dilute solution, mixing, and then lyophilizing. All other complexes were formed in this fashion except with a slight excess of the inhibitor; the complexes were purified subsequently by chromatography on Sephadex G-75 (Pharmacia). Complex-containing fractions were dialyzed against ^1H_2O and lyophilized.

Solutions Used for NMR Spectroscopy. Samples contained 0.6 μmol of protein dissolved in 0.5 mL of 0.5 M KCl in either ^1H_2O or ^2H_2O. Insoluble material was removed by centrifugation. The pH adjustments were carried out as described previously (Markley & Porubcan, 1976). The 0.3-0.5-mL samples were held in 5-mm o.d. NMR tubes (Wilmad).

NMR Spectroscopy. Preliminary experiments were conducted using ^1H NMR correlation spectroscopy at the NMR Facility for Biomedical Studies at Carnegie-Mellon University. The remaining ^1H NMR spectra were obtained at 360 MHz at the Stanford Magnetic Resonance Laboratory. Correlation software used at Stanford was written by S. L. Patt and W. W. Conover. A sweep width of 4100 Hz was scanned in 0.7 s. Spectra were digitized as 4096 data points, and 500 scans were averaged. Spectra were accumulated in 10 min. Similar line widths were obtained with spinning or nonspinning samples. However, since spinning introduced additional noise into the spectra, most spectra were obtained nonspinning. Digital filtering in the time domain was sometimes used to remove high-frequency artifacts. A line-broadening factor of 10 Hz was used in the exponential multiplication. The contribution from the leading edge of the water peak in ^1H_2O solution was removed by approximating the ^1H_2O peak by a series of straight-line segments and then subtracting this from the rapid-scan spectrum. The resulting difference spectrum was processed in the normal manner of correlation spectroscopy (Dadok & Sprecher, 1974). This procedure is illustrated in Figure 1. Similar results were obtained by subtracting the rapid-scan spectrum of ^1H_2O itself rather than approximating it by curve fitting. The reversibility of reported temperature and pH effects was verified by appropriate control experiments. Chemical shifts are reported with respect to internal DSS.

Results and Discussion

Rapid Acquisition of Spectra. A rapid-scan (4.5 kHz s^{-1}) 360-MHz ^1H NMR spectrum of the porcine trypsin-bovine trypsin inhibitor complex (PTr:BPTI) is shown in Figure 1a. The leading edge of the water peak is the dominant feature of the spectrum. After subtraction of the water component (Figure 1b), the interferogram corresponding to low-field resonances becomes apparent. The absence of ringing in the broad peaks indicates that still faster scans could be used for their accumulation. Faster scanning would lead, however, to broadening of the sharper components of the aromatic region (δ = 6-9). Normal correlation processing (Dadok & Sprecher, 1974; Fourier transformation, cross-correlation with a theoretical function with 20-Hz line width, and inverse Fourier transformation) results in the spectra shown in Figure 1c. As will be discussed later, it is useful to accumulate a wide sweep so that the areas of the peaks at extreme low field may be compared with the areas of other peaks in the spectrum.

The 360 MHz correlation ^1H NMR spectra of chymotrypsinogen A (Ctg), chymotrypsin A_δ (Ctr_δ), and porcine trypsin (PTr) all at pH 3.5 in ^1H_2O and 3.3 °C are compared in Figure 2. Continuous wave ^1H NMR spectra of Ctg (220 MHz and 360 MHz) and Ctr_δ (220 MHz) under these conditions were reported by Robillard & Shulman (1974a). The signal-to-noise of the broad low-field peaks in the present spectra, accumulated in 10 min, is comparable to that of the earlier study, which required 3.5 to 15 h of signal averaging. Rapid accumulation of spectra is desirable in studies of proteinases which are prone to self-proteolysis. The chemical shifts of the low-field peaks (δ = 18.06) are the same as reported previously. A second peak in the Ctg spectrum observed by Robillard & Shulman (1974a) at 300 MHz but not at 220 MHz appears in the 360 MHz spectrum at δ = 13.06. Porcine trypsin has a peak at δ = 17.99; the same chemical shift was reported for a peak of bovine trypsin under these conditions (Robillard & Shulman, 1974a).

Temperature Dependence. A striking feature of the extreme low-field peaks of uninhibited proteinases (Figure 2) is their line width. The peaks are much broader than the peaks visible above δ = 12. In order to test the possibility of an exchange broadening mechanism, the temperature dependence of porcine trypsin was investigated (Figure 3). The results plotted in Figure 4 show that the low-field peak k broadens as the temperature is raised from 3.3 to 31 °C. Above 36 °C, the peak

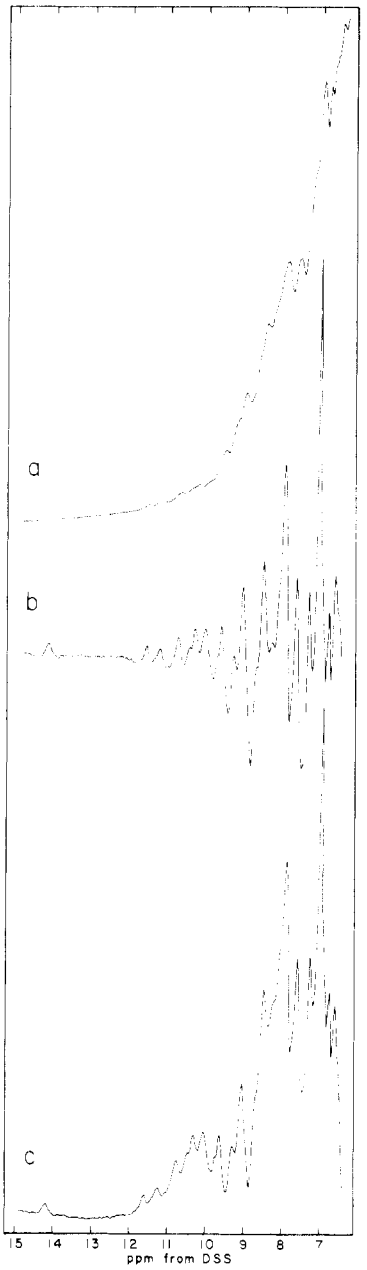

FIGURE 1: Illustration of water peak subtraction from a 360-MHz correlation ^1H NMR spectrum. Sample: 2 mM porcine trypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex in ^1H_2O containing 0.5 M KCl, pH 7.00, 30 °C. (a) Rapid scan spectrum: average of 500 4100-Hz scans, 0.7 s/scan. (b) After subtraction of water peak. (c) After correlation processing using a line-broadening factor of 20 Hz.

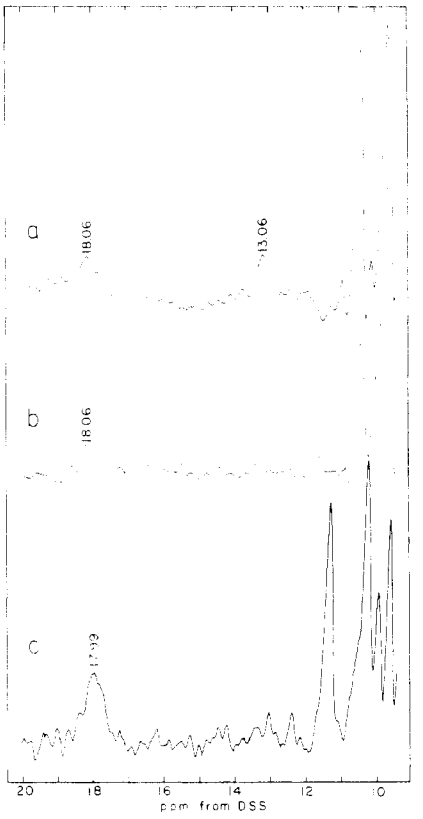

FIGURE 2: Low-field region of correlation ^1H NMR spectra at 360 MHz of (a) bovine chymotrypsinogen A, (b) bovine chymotrypsin A_δ, and (c) porcine trypsin. Samples contained 2 mM protein and 0.5 M KCl in ^1H_2O. Sample temperatures, 3.5 °C; all at pH 3.5. Spectra were averages of 500 scans (4100 Hz/0.7 s). The water peak has been subtracted out, and a 20-Hz line broadening factor was used in processing.

is no longer resolved. On the other hand, peak m shows normal temperature dependence. The broadening of peak m as the temperature is lowered is readily explained by the increase in solvent viscosity. The chemical shifts of peaks k and m are nearly independent of temperature. At the lowest temperature studied (0.3 °C) there is an anomalous increase in the width of peak k, and a second peak 1 appears at $\delta = 13.32$ (Figure 3d).

It is reasonable to assume that peak m represents a single proton since its area is equivalent to other well-resolved singlet peaks in the low-field region in several spectra (Figures 3, 4, and 10). When peak k is referenced to peak m = 1 proton, its integrated intensity is relatively constant at 0.6 ± 0.1 proton over the temperature range studied. The area of peak 1 at 0.3 °C (based on peak m = 1 proton) is 0.5 ± 0.1 proton.

These results suggest that peak k is broadened by an exchange mechanism of the type A $(k_1) \rightleftharpoons (k_{-1})$ B, where A and B indicate two forms of the enzyme. From the area of peak k, the equilibrium constant at pH 3.5 is close to 1 over the temperature range studied. If peak k is characteristic of state A, there should be a second peak having a similar width and area characteristic of state B, unless the proton is lost in that state or exchanges rapidly with bulk ^1H_2O. The sum of the areas of peaks k and 1 is 1.1 ± 0.2 protons. Thus, peak 1 is a possible candidate for the proton in state B. The fact that peak 1 appears at a much lower temperature than peak k and is broader than peak k at that temperature requires a more complicated mechanism than A $(k_1) \rightleftharpoons (k_{-1})$ B. Further experiments employing double resonance and chemical modification are needed to test the relationship between peaks k and 1.

The lifetime τ_A of state A may be estimated from the excess line broadening using the equation:

$$\tau_A = [\pi(w_A - w_0)]^{-1} \quad (2)$$

where w_A is the width at half height of peak k and w_0 is the width of the peak in the absence of exchange broadening. If the width of the "normal" N-H peak m is used for w_0, τ_k at pH 3.5 is found to vary between 1.2 ms at 31 °C and 2.1 ms at 3.3 °C. A similar analysis yields a lifetime of 1.2 ms for the state giving rise to peak 1 at 0.3 °C.

The temperature dependence of the spectrum of porcine trypsin was also investigated at pH 7.0. A poorly resolved peak appears at 3.3 °C (Figure 5b) that is not resolved at 5 °C (Figure 5a) or at 30 °C. The chemical shift of peak k at 3.3 °C (Figure 5b) ($\delta = 17.78$) is similar to that of the corresponding

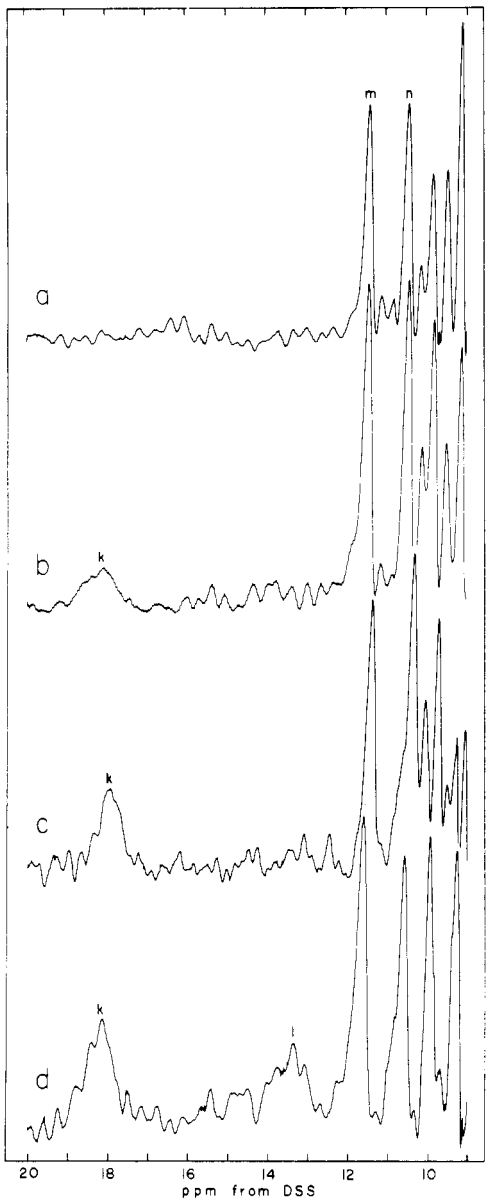

FIGURE 3: Temperature dependence of 360-MHz correlation ^{1}H NMR spectra of porcine trypsin at pH 3.52. (a) 35.9 °C, (b) 26.6 °C, (c) 3.3 °C, (d) 0.3 °C. Solutions contained 2 mM porcine trypsin and 0.5 M KCl in ^1H_2O. Spectra are averages of 500 scans (4100 Hz/0.7 s). The water peak was subtracted, and a line-broadening factor of 20 Hz was used in processing.

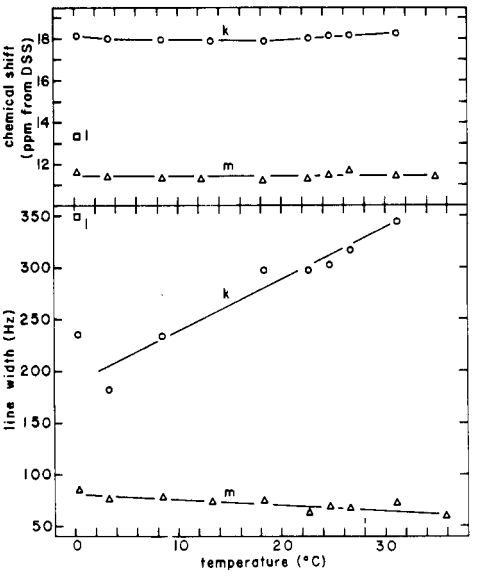

FIGURE 4: Line widths and chemical shifts (at 360 MHz) of low-field ^{1}H NMR peaks of porcine trypsin (pH 3.52) as a function of temperature. Labeling of curves follows the identification of spectra in Figure 3. A new peak, 1, appears at the lowest temperature studied, 0.3 °C. Experimental details are given in the legend for Figure 3.

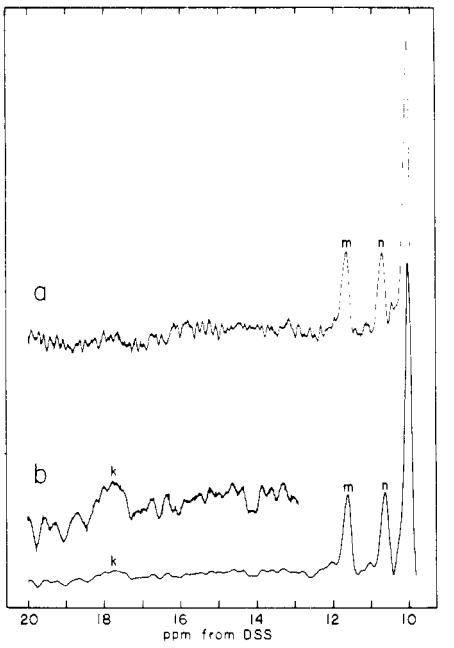

FIGURE 5: Temperature dependence of 360 MHz correlation ^{1}H NMR spectra of porcine trypsin at pH 7.00. (a) At 5 °C; (b) 3.3 °C. Solutions contained 2 mM porcine trypsin and 0.5 M KCl in ^1H_2O. Spectra are averages of 500 scans (4100 Hz/0.7 s). The water peak was subtracted, and a line-broadening factor of 20 Hz was used in processing.

peak of Ctr_δ at pH 7.0 (Robillard & Shulman, 1974a).

NMR Spectra of Proteinase:Protein Proteinase Inhibitor Complexes. Changes appear in the low-field ^{1}H NMR spectra of serine proteinases when complexes are formed between proteinases and protein proteinase inhibitors. (1) The broad peaks observable at low temperatures disappear from the spectra (Robillard & Shulman, 1974b). (2) A new N-H peak of single proton intensity appears in the 15 to 13 ppm region. This peak was reported at δ = 14.9 by Robillard & Shulman (1974b) for the Ctr_δ:BPTI complex. Spectra of a number of additional proteinase:protein proteinase inhibitor complexes have been recorded. A low-field peak is present in each complex investigated. Its chemical shift (Table I) correlates with the nature of the proteinase component of the complex but does not depend on whether the proteinase is complexed with BPTI or STI. The two inhibitors, BPTI and STI, are quite dissimilar proteins which have similar mechanisms of binding (Sweet et al., 1974; Huber et al., 1974; Bode et al., 1976b). The peak appears at approximately 14.6, 13.7, and 14.3 ppm in complexes of BCtr, BTr, and PTr, respectively (Table I). This result suggests that the peak represents an internal hydrogen bond of the proteinases that is stabilized on complex formation rather than either an internal hydrogen bond of the inhibitor or an intersubunit hydrogen bond.

In analogy with bovine anhydrochymotrypsin (Strumeyer et al., 1962; Weiner et al., 1966), porcine anhydrotrypsin differs from native trypsin only in the modification of Ser^{195} to dehydroalanine (Weidener, G. H., & Laskowski, M., Jr., personal communication). It forms a stable complex with BPTI

TABLE I: Chemical Shift of the Extreme Low-Field ^1H NMR Peak in Various Proteinase:Protein Proteinase Inhibitor Complexes at pH 7.00 in 0.5 M KCl, 30 °C.

complex	low-field peak chemical shift (ppm from DSS)
bovine chymotrypsin A_α:BPTI[a]	14.7
bovine chymotrypsinogen A:BPTI[a]	14.7
bovine chymotrypsin A_δ:STI[b]	14.5
bovine trypsin:BPTI[a]	13.6
bovine trypsinogen:BPTI[a]	13.6
bovine trypsin:STI[b]	13.8
porcine trypsin:BPTI[a]	14.2
porcine trypsin:STI[b]	14.4
porcine anhydrotrypsin:BPTI[a]	14.1

[a] Data obtained by correlation spectroscopy at 360 MHz. [b] From 250 MHz correlation spectra.

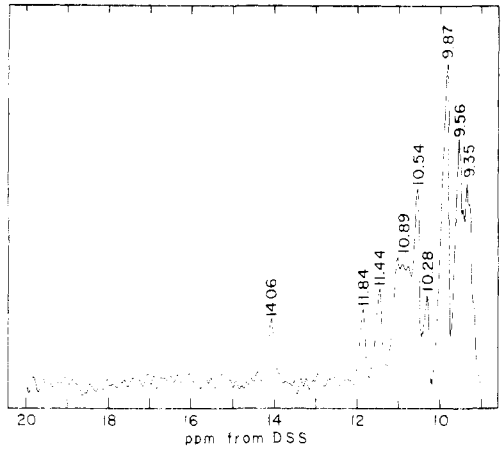

FIGURE 6: The 360-MHz correlation ^1H NMR spectrum of the porcine anhydrotrypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex in ^1H_2O containing 0.5 M KCl, pH 6.9, 30 °C. The spectrum is the average of 500 scans (4100 Hz/0.7 s). The water peak was subtracted, and a line-broadening factor of 20 Hz was used in processing.

(Weidener & Laskowski, personal communication) which, according to the bovine anhydrotrypsin:BPTI X-ray structure (Huber et al., 1975), is expected to differ from the native trypsin:BPTI complex only by the absence of a bond between Ser^{195} O^β and the carbonyl carbon of Lys^{15} of the inhibitor. A spectrum of anhydroPTr:BPTI complex at pH 7.0 and 30 °C is shown in Figure 6. The peak at $\delta = 14.06$ is present at the same chemical shift within experimental error as in the PTr:BPTI complex. In fact, all the well-reserved peaks below 10 ppm are virtually the same in the two complexes.

Recently the structures of the BTr:BPTI and BTg:BPTI complexes have been determined by X-ray crystallography and compared (Huber et al., 1974; Bode et al., 1976b; Bode & Huber, 1976). They appear to be remarkably similar except in the region of the activation peptide. ^1H NMR spectra of these two complexes at pH 7.0 and 30 °C are shown in Figure 7. There are only slight differences in the chemical shifts of all peaks below 10 ppm.

A similar comparison was made between the spectrum of the stable complex between BPTI and $BCtr_\alpha$ and the spectrum of equimolar BPTI and BCtg. Conventional methods such as gel filtration and electrophoresis do not detect complexation between chymotrypsinogen and BPTI. The spectra of these species at pH 7.0 and 30 °C are shown in Figure 8. A broad peak appears at $\delta = 14.7$ in BCtg + BPTI. However, the peak present at $\delta = 11.28$ in $BCtr_\delta$:BPTI is absent in BCtg + BPTI,

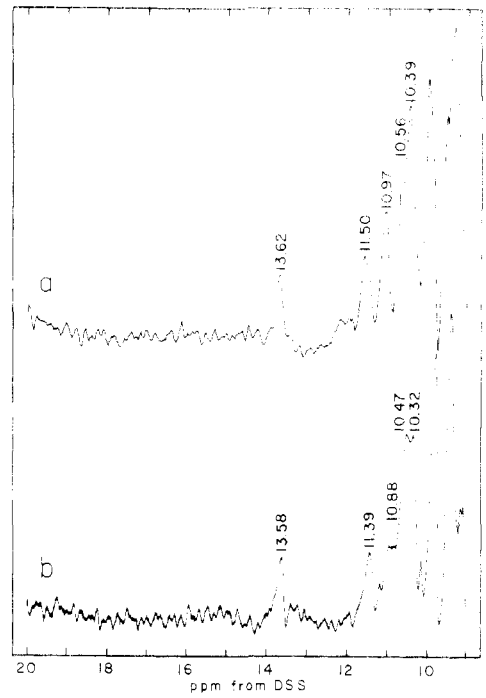

FIGURE 7: Comparison of the 360-MHz correlation ^1H NMR spectra of (a) bovine trypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex, (b) bovine trypsinogen:bovine pancreatic trypsin inhibitor (Kunitz) complex. Sample conditions: 2 mM complex in ^1H_2O with 0.5 M KCl, pH 7.0, 30 °C. Spectra are averages of 500 scans (4100 Hz/0.7 s). The water peak was subtracted, and a line-broadening factor of 20 Hz was used in processing.

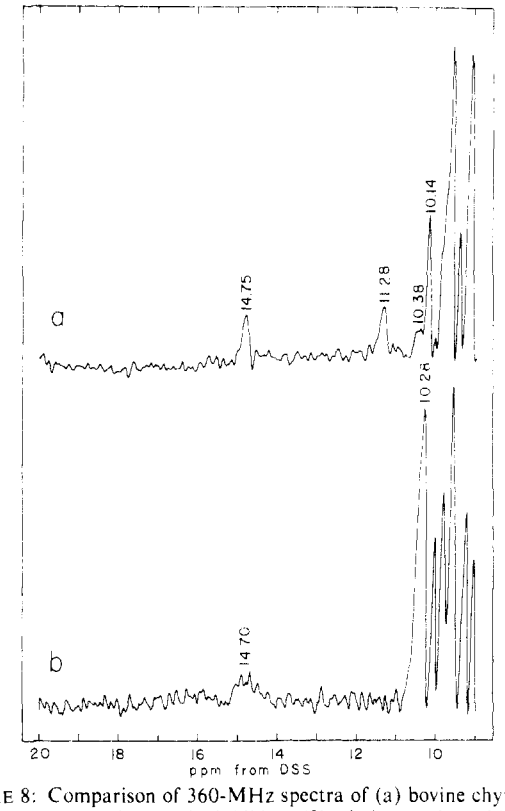

FIGURE 8: Comparison of 360-MHz spectra of (a) bovine chymotrypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex, (b) bovine chymotrypsinogen plus equimolar bovine pancreatic trypsin inhibitor (Kunitz). The broad peak at $\delta = 14.70$ in spectrum b indicates that a short-lived complex is formed between Ctg and BPTI; differences in the other N–H peaks indicate that the structures of the Ctr_α:BPTI and Ctg:BPTI complexes are different (see the text). Sample conditions: 2 mM proteins in ^1H_2O with 0.5 M KCl, pH 7.00, 30 °C. Spectra are the average of 500 scans (4100 Hz/0.7 s). The water peak was subtracted, and a line-broadening factor of 20 Hz was applied in processing.

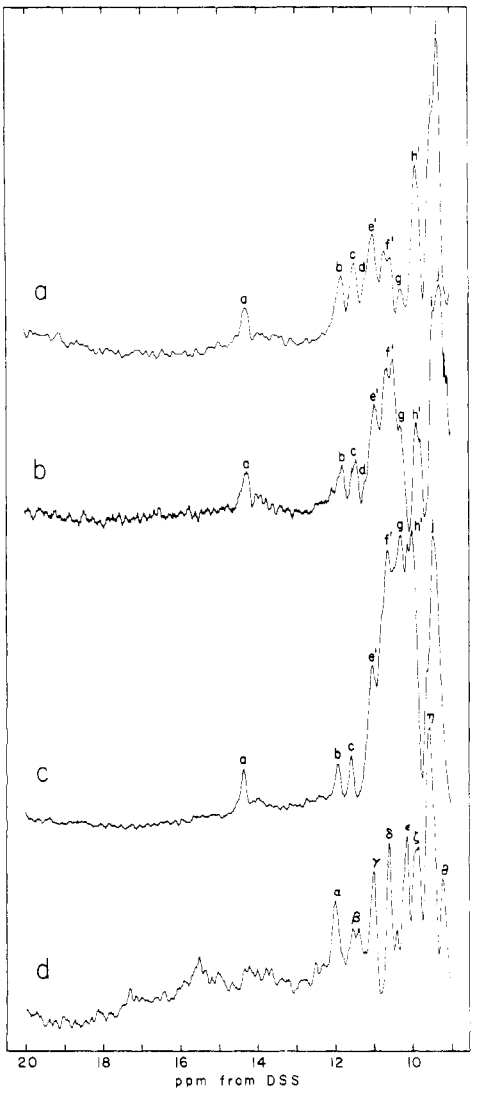

FIGURE 9: pH dependence of the low-field correlation ^1H NMR spectrum of the complex between porcine trypsin and bovine pancreatic trypsin inhibitor (Kunitz) (a) pH 6.00, (b) pH 5.01, (c) pH 4.02, (d) pH 3.00. Sample conditions: 2 mM protein in ^1H_2O with 0.5 M KCl, 30 °C. Spectra are the average of 500 scans (4100 Hz/0.7 s). The water peak was subtracted, and a line-broadening factor of 20 Hz was applied in processing.

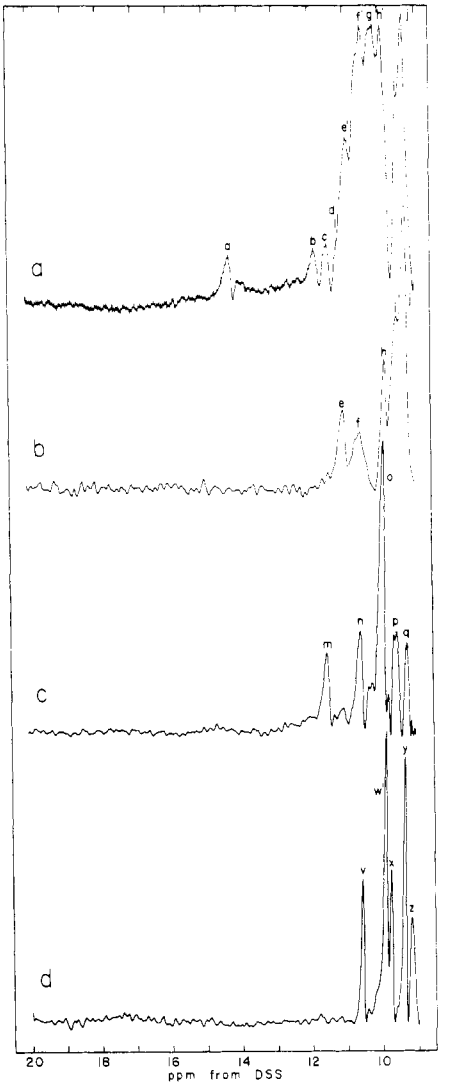

FIGURE 10: The 360-MHz correlation ^1H NMR spectra used to classify N–H peaks according to their exchange rate and protein of origin (see Table II). (a) Porcine trypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex in ^1H_2O, pH 6.99. (b) Porcine trypsin:bovine pancreatic trypsin inhibitor (Kunitz) complex prepared and lyophilized from ^1H_2O and dissolved in ^2H_2O at time zero, pH 7.12; spectrum obtained during time 5–15 min. (c) Porcine trypsin in ^1H_2O, pH 7.00. (d) Bovine pancreatic trypsin inhibitor in ^1H_2O, pH 7.00. Sample conditions: 2 mM proteins, solution contained 0.5 M KCl, 30 °C. Spectra are the average of 500 scans (4100 Hz/0.7 s). The water peak in spectra taken in ^1H_2O was removed by subtraction. A line-broadening factor of 20 Hz was applied in spectra a–c; a line-broadening factor of 10 Hz was applied in spectrum d.

and there are numerous differences in the region between 10.5 ppm and 9.0 ppm. The broad peak at δ = 14.7 in (BCtg + BPTI) apparently indicates complexation. The lifetime of the complex is estimated to be 1.3 ms by eq 2 using the width of the δ = 14.7 peak of BCtr:BPTI (90 Hz) for w_0. An estimate of the dissociation constant K_d for the BCtg:BPTI complex may be made if one assumes that the rate of formation of the complex is similar to that of the BCtr:BPTI complex, which is approximately 4×10^5 s^{-1} M^{-1} (Engel et al., 1974). The calculated value is $K_d = 2 \times 10^{-3}$ M. Differences in the ^1H NMR peaks above 12 ppm (Figure 8) indicate that the structure of this weak complex is significantly different from the much stronger $BCtr_\alpha$:BPTI complex.

pH Dependence of PTr:BPTI. Spectra of PTr:BPTI at selected pH values are shown in Figure 9. There are no significant changes between pH 7.0 and 5.0. At pH 4.0, the positions of peaks a, b, and c are unchanged, but peak d has moved, and there are some minor changes in the region between 9 ppm and 11 ppm. A gross alteration in the spectrum appears between pH 4.0 (Figure 9c) and pH 3.0 (Figure 9d). Peaks a, b, and c are missing, and there is a major change in the peaks between 12 ppm and 4 ppm. An abrupt increase in K_d of the complex at low pH is well known (Finkenstadt et al., 1974); however, at the concentration used in the present study a complex would still be present. Potentiometric techniques indicate that the low pH transition involves the cooperative binding of three protons (Finkenstadt et al., 1974). A ^1H NMR study of the histidine $C^{\epsilon 1}$–H resonances of porcine trypsin in this complex indicated that two of the protons add to active site components with a pH_{mid} of 3.5 (Markley & Porubcan, 1976).

Origin and Deuterium Exchange Properties of the Hydrogen-Bonded Protons. As an approach to the assignment of the low-field peaks of the PTr:BPTI complex, the resonances may be classified as arising from: (1) H bonds of PTr unchanged upon complexation; (2) H bonds of BPTI unchanged upon complexation; (3) new or altered H bonds resulting from complexation. For example, peak a (Figures 9 and 10) has been assigned to the third category. It is also of interest to classify

TABLE II: Low-Field N-H ^1H NMR Peaks in Spectra of Bovine Pancreatic Trypsin Inhibitor (BPTI), Porcine Trypsin (PTr), and Their Complex (PTr:BPTI).

protein	solvent	peak[a]	estimated no. of protons	chemical shift	exchange-ability[b]	origin of peak in complex
BPTI[c]	^2H_2O	v	1	10.60	slow	
		w	1	9.98	slow	
		x	1	9.82	slow	
		y	2	9.41	slow	
		z	1	9.24	slow	
BPTI	^1H_2O	v	1	10.53	slow	
		w	~3	9.99	2 fast, 1 slow	
		x	1	9.72	slow	
		y	2	9.34	slow	
		z	1	9.13	slow	
PTr[e]	^2H_2O	q	~1	9.22	slow	
PTr	^1H_2O	m	1	11.51	fast	
		n	1	10.56	fast	
		o	~4	9.86	fast	
		p	1	9.58	fast	
		q	~1	9.24	slow	
PTr:BPTI	^2H_2O	e	1	10.98	slow	new in complex
		f'	1	10.51	slow	BPTI (v)
		h'	1	9.81	slow	BPTI (x)
		i	~1	9.49	slow	PTr (p)
		j	~4	9.28	slow	PTr (q)
PTr:BPTI	^1H_2O	a	1	14.22	fast	new in complex
		b	1	11.76	fast	new in complex
		c	1	11.40	fast	PTr (m)
		d	~1	11.22	fast	new in complex
		e'	≥1	10.84	slow	new in complex
		f'	*d*	10.46	fast	new in complex
					slow	BPTI (v)
PTr:BPTI	^1H_2O	g	*d*	10.19	fast	new in complex
		h'	*d*	9.86	slow	BPTI (x)
					fast	PTr (o)
		i	*d*	9.27	slow	BPTI (y)
		j	*d*	9.23	slow	BPTI (z)
					slow	PTr (q)

[a] Letters designating peaks correspond to those in Figure 9. [b] Exchangeability on dissolving the protein in ^2H_2O: fast, $t_{1/2} \ll 10$ min; slow, $t_{1/2} \gg 10$ min. [c] Data from Masson & Wüthrich (1973). [d] Intensity not measured because of uncertainty in the baseline. [e] From Markley & Porubcan (1976).

the resonances on the basis of the deuterium exchange kinetics of the protons giving rise to the NMR peaks.

The results of such classification experiments are presented in Figure 10 and Table II. All resonances in the region below 9 ppm correspond to exchangeable (not carbon-bound) hydrogens. These peaks are missing in ^1H NMR spectra of PTr (Markley & Porubcan, 1976) or BPTI (Karplus et al., 1973; Masson & Wüthrich, 1973) preexchanged in ^2H_2O at elevated temperature or pH. Peak q of PTr (Figure 10c) and peak y and half the intensity of peak w' of BPTI (Figure 10d) exchange very slowly in ^2H_2O (Masson & Wüthrich, 1973). Spectra of PTr and BPTI in ^1H_2O at 30 °C, pH 7.0, are shown, respectively, in Figures 10c and 10d. Additional peaks appear that represent protons that exchange slowly with bulk water. When the PTr:BPTI complex is formed in ^1H_2O, the spectrum shown in Figure 10a results. Note that the spectrum of the complex contains peaks not found in the sum of the spectra of the two individual protein components. These new peaks correspond to hydrogen bonds (or otherwise hindered exchangeable protons) characteristic of the complex. It is significant that peaks corresponding to all the peaks of both isolated components are present in the complex. That is, there is no evidence for any interactions in PTr or BPTI being broken when the complex is formed. This result is consistent with the idea that a rigid proteinase combines with a rigid inhibitor and that there is little, if any, induced fit (Huber et al., 1974).

The spectrum shown in Figure 10b was obtained by dissolving in ^2H_2O PTr:BPTI complex lyophilized from ^1H_2O. Accumulation of the spectrum was started 5 min after the protein was dissolved and completed 10 min later. Since peak a is not present in the spectrum, its half-time for exchange must be less than 4×10^2 s. This is two orders of magnitude shorter than the dissociation half-time for the complex (7×10^4 s; Finkenstadt et al., 1974).

Conclusion

Hunkapiller et al. (1973) were unable to resolve the low-field N-H peaks of chymotrypsinogen A and chymotrypsin A_δ reported by Robillard & Shulman (1972). They attempted to reproduce the spectra at three different frequencies using Varian XL-100-15, HR-220, and HR-300 spectrometers. The present work at 250 and 360 MHz (Figure 2) is in agreement with the original experimental results of Robillard & Shulman at those pH values studied.[2]

[2] The low-field N-H peaks also have been resolved using the "2-1-4" sequence [Redfield, A. G., Kung, S. D., & Ralph, E. K. (1975) *J. Magn. Reson. 19*, 114] (W. M. Westler & J. L. Markley, unpublished).

The Question of Assignments. The complete interpretation of these results hinges on the correct assignment of peaks to specific hydrogen bonds in the proteins and protein complexes. The only assignments postulated to date are those of Robillard & Shulman (1974a,b) for the peak at lowest field in zymogens, proteinases, and proteinase:proteinase inhibitor complexes. Rigorous tests of these assignments as well as assignments of other peaks will require further experiments. At present it may be useful to discuss the evidence for the Robillard & Shulman assignments and the implications of these assignments.

The broad, low-field peak of chymotrypsinogen A was attributed to an His N-H on the basis of its exchange properties and histidine-like titration curve. Since chymotrypsinogen has two histidine residues, specific assignment to His^{57} was adduced from the similarity of chemical shifts of the low-field peaks in chymotrypsin and chymotrypsinogen. In other words, the assignment of the peak to His^{57} in chymotrypsinogen was based on the assignment of a similar peak in chymotrypsin. The assignment of the peak in chymotrypsin resulted from experiments which showed that alkylation of His^{57} or Ser^{195} changes the pH dependence of the chemical shift. The assignment of the peak in the complex rests solely on the observation that the new peak appears in the region of the spectrum formerly occupied by the broad peak of chymotrypsin A_δ at high pH. On the strength of these results Robillard & Shulman (1974a,b) reached the three following conclusions: (i) The hydrogen-bonded charge relay system has the same structure in chymotrypsin and chymotrypsinogen. (ii) The pK of His^{57} in chymotrypsinogen A is the same as that of His^{57} in chymotrypsin A_δ (pK = 7.5 at 3 °C). (iii) His^{57} of chymotrypsin does not pick up a proton on formation of the complex with BPTI.

While it seems clear that the low-field ^{1}H-NMR peaks monitor changes occurring at the active sites of serine proteinases, their assignment to the His^{57}–Asp^{102} hydrogen bond and/or the resulting conclusions are not readily compatible with certain data available at present.

(1) Two recent papers have presented evidence that Asp^{102} rather than His^{57} of serine proteinases has the pK' near 7 (Koeppe & Stroud, 1976) and that the pK' of His^{57} is 3.3 or lower (Hunkapiller et al., 1973). On the other hand, ^{1}H NMR studies from this laboratory on His $C^{\epsilon 1}$ protons support the conclusion of Robillard & Shulman (1972) that the pK' of His^{57} is higher than that of Asp^{102} (Markley & Porubcan, 1976; Markley & Ibañez, 1978; Porubcan et al., 1978). The controversy concerning the relative pK' values may have little bearing on the assignment of the N-H to a hydrogen bond between His^{57} and Asp^{102} since the chemical shift of the N-H would be affected by protonation of either residue.

(2) Refinement of the X-ray structures of chymotrypsinogen and chymotrypsin indicates a difference between the structure of the catalytic triad in the zymogen and the enzyme. In the zymogen, there appears to be a normal hydrogen bond between the Ser^{195} hydroxyl and His^{57}; in the enzyme, the Ser^{195}–His^{57} hydrogen bond is highly distorted or more probably absent (Birktoft et al., 1976; Matthews et al., 1977). From these results one expects a different pK' (and perhaps also a different chemical shift) for the His^{57} N-H in the zymogen and the enzyme. Other NMR studies bear this out. The ^{1}H NMR chemical shift of the His^{57} $C^{\epsilon 1}$-H peak is different in chymotrypsinogen A and chymotrypsin A_α; and the pK' of His^{57} at 31 °C in ^2H_2O is 7.3 for chymotrypsinogen A and 6.1 for chymotrypsin A_α (Markley & Ibañez, 1978). Similar chemical shift differences are observed in porcine trypsinogen and porcine trypsin; the pK' of His^{57} is 7.5 in the zymogen and 5.0 in the enzyme (Markley & Porubcan, 1976; Porubcan et al., 1978). ^{31}P NMR studies of diisopropylphosphoryl Ser^{195} BCtg and BCtr show that the phosphate triester has a different environment in the zymogen and activated enzyme derivatives (Reeck et al., 1977).

In defense of the assignments, the similarity of the pK's of the low-field N-H peaks of BCtg and $BCtr_\delta$ (Robillard & Shulman, 1974a) may result from different temperature coefficients for the pK's of His^{57} in the enzyme and the zymogen which make the pK's equal at 3 °C but unequal at 31 °C. The pK value of 7.5 for BCtr at 3 °C is in good agreement with the thermodynamic results of Hanai (1976) for an active site residue. The similarity of chemical shifts of the low-field N-H peaks in BCtg and $BCtr_\delta$ may simply reflect that the Asp^{102}–His^{57} hydrogen bond is very similar in the zymogen and the enzyme (Birktoft et al., 1976), and is not influenced by other structural changes. Robillard & Shulman did report an unexplained inflection in the titration curve of the enzyme at pH 5.7 that was not present in the zymogen.

(3) The structure of the bovine trypsin:BPTI complex obtained by X-ray crystallography (Bode et al., 1976b; Huber et al., 1974) indicates that the C^0 of Lys^{16} of BPTI is tetrahedrally distorted and that there is a partial bond between this atom and the Ser^{195} O^γ of trypsin. The Ser^{195} O^γ is within H-bonding distance of the $N^{\epsilon 2}$ of His^{57} (2.7 Å). Huber & Bode (1978) show the proton still on the Ser^{195} oxygen. ^{1}H NMR studies in ^2H_2O at 30 °C of the $C^{\epsilon 1}$-H of His^{57} in the complexes PTr:BPTI (Markley & Porubcan, 1976) and $BCtr_\delta$:BPTI (Markley & Ibañez, 1978) indicate that His^{57} supports at least a partial positive charge in the complexes.

In order to reconcile the data of Robillard & Shulman (1974b) with a complex having a positively charged His^{57} (Markley & Ibañez, 1978; Markley & Porubcan, 1976), one has to postulate that the chemical shift of the N-H proton is as responsive to environmental effects as it is to the protonation state of His^{57}. If the assignments of peaks 1 and m of porcine trypsin (Figure 3) to His^{57} in different conformational states prove correct, the conflict can be resolved by postulating that the conformation giving rise to peak 1 is the one stabilized in the complex. In this case, the chemical shift of the N-H peak in the complex corresponds to that of protonated His^{57} in agreement with the tetrahedral mechanism.

The intensity and temperature dependence of the extreme low-field peaks of chymotrypsin and chymotrypsinogen need to be reinvestigated in light of the present results with porcine trypsin. The spectra in Figure 2 suggest that the peaks at δ = 18 of chymotrypsinogen and chymotrypsin also have fractional proton intensity and are exchange broadened. The areas of the two peaks of chymotrypsinogen at δ = 18.06 and 13.06 (Figure 2) sum to an intensity close to that of one of the sharp singlet peaks at higher field. This suggests that the two broad peaks may correspond to the same proton in different conformational states. Matthews et al. (1977) have discussed a possible flip-flop model for His^{57} of serine proteinases in which there is alternate binding of the His^{57} $N^{\delta 1}$-H to the $O^{\delta 1}$ and $O^{\delta 2}$ oxygens of Asp^{102}. It has been postulated that a flip-flop mechanism may be catalytically important and may be required to explain kinetic results on aminolysis of acylchymotrypsin (Satterthwait & Jencks, 1974). It is possible that each of the broadened fractional proton peaks represents one of these two forms. If so, the chemical shift of the Asp^{102}–His^{57} H-bonded proton would depend on the orientation of the His^{57} ring as well as the protonation states of His^{57} and Asp^{102}. The X-ray data show that, in the bovine trypsin:bovine pancreatic trypsin inhibitor complex (Rühlman et al., 1973; Bode et al., 1975), the His^{57} $N^{\epsilon 2}$ is directed toward the Ser^{195} O^γ, whereas in aromatic boronic acid:subtilisin complexes the His^{57} $N^{\epsilon 2}$ is directed

toward the location of the substrate's leaving group (Matthews et al., 1975, 1977). The results suggest that one of the conformational forms observed by ^1H NMR spectroscopy may be stabilized in BPTI complexes, and the other in aromatic boronic acid complexes. According to the arguments above, the chemical shift of the N-H in aromatic boronic acid complexes should be downfield from the 13.6–14.7 ppm range observed in BPTI complexes (Table I). Indeed this is what Robillard & Shulman (1974b) found for the low-field N-H peak in compiexes between chymotrypsin A_δ and benzene boronic acid (16.3 ppm) and chymotrypsin A_δ and 3-phenylethylboronic acid (17.2 ppm).

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