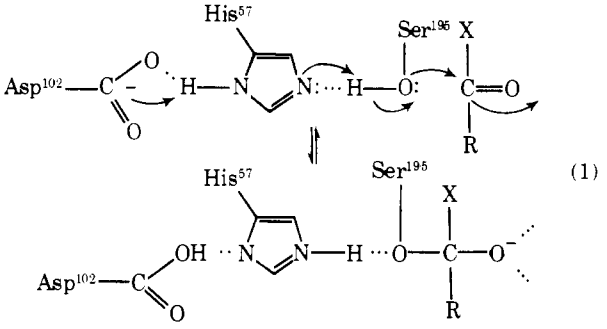
Zymogen Activation in Serine Proteinases. Proton Magnetic Resonance pH Titration Studies of the Two Histidines of Bovine Chymotrypsinogen A and Chymotrypsin A_α†

John L. Markley*,‡ and Ignacio B. Ibañez

ABSTRACT: Reversible unfolding of bovine chymotrypsinogen A in ^2H_2O either by heating at low pH or by exposure to 6 M guanidinium chloride results in the exchange of virtually all the nitrogen-bound hydrogens that give rise to low-field ^1H NMR peaks, without significant exchange of the histidyl ring $C^{\epsilon 1}$ hydrogens. These preexchange procedures have enabled the resolution of two peaks, using 250-MHz correlation ^1H NMR spectroscopy, that are attributed to the two histidyl residues of chymotrypsinogen A. Assignments of the $C^{\epsilon 1}$ hydrogen peaks to histidine-40 and -57 were based on comparison of the NMR titration curves of the native zymogen with those of the diisopropylphosphoryl derivative. Two histidyl $C^{\epsilon 1}$ H peaks were also resolved with solutions of preexchanged chymotrypsin A_α. The histidyl peaks of chymotrypsin A_α were assigned by comparison of NMR titration curves of the free enzyme with those of its complex with bovine pancreatic trypsin inhibitor (Kunitz). The NMR titration curves of histidine-57 in the zymogen and enzyme and histidine-40 in the zymogen exhibit two inflections; the additional inflections were assigned to interactions with neighboring carboxyl groups: aspartate-102 in the case of histidine-57 and aspartate-194 in the case of histidine-40 of the zymogen. In bovine chymotrypsinogen A in ^2H_2O at 31 °C, histidine-57 has a pK' of 7.3 and aspartate-102 a pK' of 1.4, and the histidine-40–aspartate-194 system exhibits inflections at pH 4.6 and 2.3. In bovine chymotrypsin A_α under the same conditions, the histidine-57–aspartate-102 system has pK' values of 6.1 and 2.8, and histidine-40 has a pK' of 7.2. The results suggest that the pK' of histidine-57 is higher than the pK' of aspartate-102 in both zymogen and enzyme. A significant difference exists in the structure and properties of the catalytic center between the zymogen and activated enzyme. In addition to the difference in pK' values, the chemical shift of histidine-57, which is highly abnormal in the zymogen (deshielded by 0.6 ppm), becomes normalized upon activation. These changes may explain part of the increase in the catalytic activity upon activation. The ^1H NMR chemical shift of the $C^{\epsilon 1}$ H of histidine-57 in the chymotrypsin A_α–pancreatic trypsin inhibitor (Kunitz) complex is constant between pH 3 and 9 at a value similar to that of histidine-57 in the porcine trypsin–pancreatic trypsin inhibitor complex [Markley, J. L., and Porubcan, M. A. (1976), *J. Mol. Biol. 102*, 487–509], suggesting that the mechanisms of interaction are similar in the two complexes.

Bovine chymotrypsinogen contains two histidyl residues, both of which play significant roles. One histidyl residue, His^{40},[1] is affected by zymogen activation (Freer et al., 1970; Kraut, 1971; Wright, 1973a). In chymotrypsinogen, His^{40} is hydrogen bonded to Asp^{194}, which is adjacent to the active site. Upon activation to chymotrypsin, Asp^{194} pivots around and forms a salt linkage with the newly formed amino-terminal Ile^{16}, and His^{40} hydrogen bonds instead to the carbonyl of Gly^{193} (Matthews et al., 1967). The new interaction between Asp^{194} and Ile^{16} is considered to be critical for establishing and maintaining the active conformation of the enzyme (Matthews et al., 1967; Oppenheimer et al., 1966). The inactivation of chymotrypsin at high pH (Himoe et al., 1967) is attributed to deprotonation of the Ile^{16} α-amino group which abolishes this salt linkage.

The other histidine, His^{57}, is essential for catalytic activity (Schoellman and Shaw, 1963). It forms a part of the "charge-relay" system (Blow et al., 1969), which appeared to be a hydrogen-bonded structure consisting of His^{57}, the buried carboxyl of Asp^{102}, and the hydroxyl of the active site Ser^{195}. Blow et al. (1969) originally proposed a catalytic mechanism involving formation of a serine alkoxide ion. The mechanism generally accepted at present consists of a concerted movement of electrons and two proton shifts (Hunkapiller et al., 1973, 1976) leading to the transfer of the negative charge from Asp^{102} to an oxyanion formed at the substrate carbonyl.



This mechanism is controversial, since there also is evidence for the existence of a His^{57}–Asp^{102} ion pair (Schotton et al., 1971; Robillard and Shulman, 1972, 1974a,b; Tulinsky et al., 1973a; Rogers and Bruice, 1974; Delbaere et al., 1975; Porubcan and Markley, 1976; Huber and Bode, 1977). His^{57}, Asp^{102}, and Ser^{195} are conserved in sequences of all serine proteinases related to chymotrypsin (Blow et al., 1969), and

†From the Biochemistry Division, Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received January 27, 1977; revised manuscript received April 24, 1978.* Preliminary accounts of this research have been presented (Porubcan et al., 1975, 1977; Ibañez et al., 1976a,b). Supported by National Institutes of Health Grants GM 19907 to the Purdue Research Foundation and RR 00292 to the NMR Facility for Biomedical Studies, Carnegie-Mellon University, Pittsburgh, Pa. (where the 250-MHz spectroscopy was carried out).

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[1] The chymotrypsinogen numbering system is used throughout to specify residues in this and related serine proteinases.

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a similar hydrogen-bonded arrangement of these three residues has been found by X-ray crystallography in the homologous proteins, bovine trypsin (Stroud et al., 1974; Bode and Schwager, 1975; Bode et al., 1976), porcine elastase (Shotton and Watson, 1970), *Streptomyces griseus* proteinase B (Delbaere et al., 1975), and α-lytic proteinase (M. James, personal communication). The discovery by X-ray crystallography of a similar Asp-His-Ser hydrogen-bonded system at the active site of subtilisin (Wright et al., 1969), a bacterial serine proteinase evolutionarily unrelated to the mammalian chymotrypsin family (Markland and Smith, 1967), suggests that this structure has features of particular catalytic importance.

The initial X-ray study of chymotrypsinogen indicated that the Asp-His-Ser triad has nearly the same configuration in the zymogen as in the activated enzyme (Freer et al., 1970). Minor differences in this region were observed, but the changes did not appear to be of importance for the proposed catalytic mechanism (Freer et al., 1970; Kraut, 1971; Wright, 1973a). This conclusion was also supported by the pioneering ^1H NMR studies of Robillard and Shulman (1972, 1974a). A measurable activity for chymotrypsinogen was determined recently which is 10^{-6}-10^{-7} that of activated enzyme (Morgan et al., 1972; Gertler et al., 1974). A large part of the increased enzymatic activity upon activation was attributed at that time to the generation of a better substrate binding site rather than to a change in the catalytic groups per se (Gertler et al., 1974; Wright, 1973b). Recently, detailed comparisons have been made of several X-ray structures of serine proteinases which indicate that the Asp·His interaction remains the same upon zymogen activation but that the His·Ser hydrogen bond which is normal in chymotrypsinogen is either bent and weak in chymotrypsin or more likely not present at all (Matthews et al., 1977; Birktoft et al., 1976).

Subsequent to the early studies of histidyl residues in bovine pancreatic ribonuclease A (Bradbury and Scheraga, 1966; Meadows et al., 1967) and other small proteins by nuclear magnetic resonance spectroscopy, it has been clear that similar investigations of serine proteinases would be of considerable interest. Early attempts at resolving NMR peaks corresponding to the ring $C^{\epsilon 1}$ proton of histidyl residues in serine proteinases were unsuccessful (Bradbury and Wilairet, 1967), and resolution of an immobilized, hydrogen-bonded histidine in a protein the size of chymotrypsin was judged to be practically impossible on theoretical grounds (Bradbury et al., 1971). We have found that the severe difficulty in resolving the histidyl $C^{\epsilon 1}$ proton NMR peaks of these proteinases is caused by interference from the background of peaks corresponding to very slowly exchangeable N-*H* groups. If these peaks are removed by deuterium exchange under conditions that do not lead to deuterium exchange of the histidine $C^{\epsilon 1}$ protons, the spectrum may be simplified sufficiently so that the histidyl peaks can be resolved. Adequate preexchange may be attained either by reversible heat denaturation of the protein molecules at low pH in ^2H_2O or by reversible unfolding by guanidinium chloride in ^2H_2O. Conditions have been optimized so that there is no appreciable loss of enzyme activity as a result of the exchange process. Following preexchange in ^2H_2O, we have succeeded in resolving NMR peaks corresponding to all the histidyl residues of porcine trypsin (Porubcan et al., 1976), bovine and porcine trypsinogen (Porubcan et al., 1978), and α-lytic proteinase (Westler and Markley, 1978). We report here the NMR titration curves for the two histidyl residues of bovine chymotrypsinogen A and bovine chymotrypsin A_α and the assignment of these curves to His^{40} and His^{57}; also, we discuss the observed pH transitions affecting these residues. The results indicate that zymogen activation produces significant changes in the chemical properties of the catalytic residues which may play an important role in the amplification of enzyme activity. We hope to demonstrate that the pK' of His^{57} is higher than the pK' of Asp^{102} in both the zymogen and enzyme. Hence, the mechanism shown in eq 1 involving a concerted transfer of two protons probably is incorrect.

Experimental Section

Materials. Bovine chymotrypsinogen A (five times crystallized, grade CGC) and bovine trypsin (three times crystallized, grade TRL 3) were from Worthington Biochemical Corp. Bovine pancreatic trypsin inhibitor (BPTI)[2] (Trasylol, registered trademark of Bayer AG) was a generous gift from Bayer AG. Deuterium oxide (^2H_2O), 99.8% isotopically pure, was purchased from Bio-Rad Laboratories and Mallinckrodt Chemical Co. Potassium deuteroxide was from Merck of Canada, and deuterium chloride was from Diaprep. N^α-Tosyl-L-lysyl chloromethyl ketone (TLCK) and *p*-nitrophenyl *N*-acetylbenzylcarbazate (NPABC) were from Nutritional Biochemical Corp., and *p*-nitrophenyl acetate (NPA) and UltraPure Tris buffer were from Schwarz/Mann Biochemicals. Diisopropyl phosphofluoridate (DFP) was purchased from Aldrich. All other chemicals used were reagent grade or better.

Assays of Chymotrypsin Activity. Chymotrypsin activity was measured by the ATEE assay (Wilcox, 1970) using a Radiometer automatic titrator or by a variation of the NPA assay of Spencer and Sturtevant (1959). For the latter assay, 0.5 mL of the enzyme solution was mixed with 0.5 mL of a 2.0×10^{-4} M Tris buffer (pH 7.2), 1% in ethanol. The absorbance at 400 nm was measured vs. time on a Beckman Model 24 spectrophotometer. The number of active sites was calculated from the absorbance extrapolated to zero time. The samples typically contained 80% active sites.

Preparation of Deuterium-Exchanged Protein Samples. Bovine chymotrypsinogen A (BCtg) was heated in ^2H_2O at pH* 3.0 for 60 min at 60 °C. After cooling, the pH* was brought to 7.5. Any precipitated protein present was removed by centrifugation, and the supernatant was lyophilized. Five to ten percent of the original specific activity was lost by this procedure. The product was lyophilized and stored at −20 °C until use. The preexchanged chymotrypsinogen A is designated BCtg(pre-exch). Equivalent exchange of NH groups was achieved by dissolving chymotrypsinogen in 6 M guanidinium chloride in ^2H_2O and exchanging at pH* 3.0 for 12 h at 25 °C. The samples were desalted by ultrafiltration on UM membranes (Amincon) followed by gel filtration on a Sephadex G-25 column. Samples prepared in this manner had normal activity after trypsin activation.

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[2] Abbreviations used are: TLCK, N^α-tosyl-L-lysyl chloromethyl ketone; ATEE, acetyl-L-tyrosine ethyl ester; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NPA, *p*-nitrophenyl acetate; DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl; BCtg, bovine chymotrypsinogen A; $BCtr_\delta$, bovine chymotrypsin A_δ; BCtg(pre-exch), bovine chymotrypsinogen A preexchanged in ^2H_2O solution in order to deuterate slowly exchangeable groups; BCtr(pre-exch), BCtg(pre-exch) activated by bovine trypsin in ^2H_2O; BPTI, bovine pancreatic trypsin inhibitor (Kunitz); BPTI(pre-exch), BPTI preexchanged in ^2H_2O in order to deuterate slowly exchangeable groups; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate. The symbol pH* is used to indicate the uncorrected pH meter reading of ^2H_2O solutions obtained using a glass electrode standardized with buffers made up in ^1H_2O. Notation used follows the "Recommendations for the Presentation of NMR Data for Publication in Chemical Journals" [*Pure Appl. Chem.* 45, 219 (1976)]. Because of differences in the numbering of the histidyl ring in the chemical and biochemical literature, the crystallographic convention is utilized here: $C^{\epsilon 1}$ is C(2) in the usual biochemical notation; $N^{\delta 1}$ is N_π; and $N^{\epsilon 2}$ is N_τ.

$NaDodSO_4$ gels indicated that the chymotrypsinogen contained a small amount of lower molecular weight protein, nearly all of which could be removed by chromatography on a Sepharose-BPTI column. This chromatography step did not alter the appearance of low-field ^1H NMR spectra of BCtg(pre-exch) but did increase its long-term stability in solution.

BCtg(pre-exch) was activated by incubation for 60 min with 3 mol % trypsin in ^2H_2O at pH 7.5 and 25 °C (Garel and Labouesse, 1973). The remaining tryptic activity was removed by the addition of TLCK (Shaw et al., 1965). The product was lyophilized at neutral pH and stored at −20 °C for later use. The activated preexchanged product is designated as BCtr(pre-exch). $NaDodSO_4$ gels of the commercial chymotrypsinogen A_δ and that prepared from BCtg(pre-exch) were similar and indicated that the preparations contained a significant amount of a two-chain contaminant. Miller et al. (1971) found that the product of "fast activation" contains, in addition to chymotrypsin A_δ, a 25% contaminant with a Thr amino terminal. Homogeneous chymotrypsin A_δ was prepared by activating for 20 min at 5 °C. Chymotrypsin A_α (Worthington) was preexchanged directly according to the procedure described above for chymotrypsinogen. Preexchange of chymotrypsin A_α in ^2H_2O leads to a loss in specific activity of 20% or less.

Bovine pancreatic trypsin inhibitor (BPTI) was exchanged by heating 7 mg/mL in ^2H_2O at pH 3.0 for 80 min at 80 °C. The preexchanged product is designated BPTI(pre-exch). Previous experiments demonstrated that this procedure removes all NMR resonances from the histidine $C^{\epsilon 1}$-H region (Masson and Wüthrich, 1973; Markley and Porubcan, 1976).

Preparation of the Chymotrypsin-Pancreatic Trypsin Inhibitor Complex. Typically, 25 mg of BCtr(pre-exch) was dissolved in 10 mL of ^2H_2O. The solution was titrated with BPTI(pre-exch) dissolved in ^2H_2O until there was no remaining chymotryptic activity, as detected by the NPA assay. The complex was then lyophilized.

Preparation of DIP Derivatives. BCtg(pre-exch) was reacted with DFP at room temperature in 0.1 M Tris-^2HCl buffer in ^2H_2O (pH* 8.0) by a procedure analogous to that used by Gertler et al. (1974). The chymotrypsinogen concentration was 5.4 mg/mL. Sufficient 1 M DFP in isopropyl alcohol was added to give a DFP concentration of 2×10^{-2} M in the reaction mixture. After 20 h, the pH* was lowered to 3.0, and the insoluble material was removed by centrifugation. The protein in the supernatant was concentrated by ultrafiltration and was passed through a Sephadex G-25 column equilibrated with ^2H_2O. The protein peak was then lyophilized. Two samples were prepared by this procedure. One sample showed no detectable activity by the NPA assay after trypsin activation; the other showed 15% of the normal activity. Minor peaks were detected in the ^1H NMR spectra of the second sample which had chemical shifts identical to chymotrypsinogen A; these were absent from the first sample. BCtr(pre-exch) was reacted with DFP for 1 h with all other conditions the same.

Solutions Used for NMR Spectroscopy. Samples contained 25 mg of lyophilized chymotrypsinogen or chymotrypsin in 0.5 mL of 0.5 M KCl in ^2H_2O. Insoluble material was removed by centrifugation. The pH adjustments were carried out as described previously (Markley and Porubcan, 1976).

The pH* was measured at 25 °C before and after spectra were taken, and the spectra were used only if these measurements agreed within 0.05 pH unit. Autodigestion was easily detected in the unbuffered samples, since it led to a drift in pH. Samples exhibiting systematic pH drifts were discarded. Fresh samples were used to check individual points at high pH.

Purification of the ^2H_2O used in these studies was judged not to be necessary, since equivalent spectra were obtained with proteins dissolved in untreated ^2H_2O and in ^2H_2O purified by passage through a Chelex (Bio-Rad) column.

NMR Spectroscopy. ^1H NMR spectra were obtained in 15 min at 30 °C using the correlation technique at 250 MHz as described previously (Markley and Porubcan, 1976). The reversibility of all titration phenomena was established experimentally. All titrations were reversible with the exception of the protein precipitations which occurred at the lowest pH values studied.

The data analysis was as described previously (Markley and Porubcan, 1976). All chemical shifts are given in parts per million (ppm) from internal DSS. The chemical shifts are related to those reported previously, which were referenced to external 5% $(CH_3)_4Si$ by the equation:

$$\delta_{5\%(CH_3)_4Si} = \delta_{DSS} - 0.33 \quad (2)$$

Resolution of certain spectra was enhanced by processing data in the time domain by the optimum filtering function (Ernst, 1966) prior to Fourier transformation into the frequency domain.

Results

Preexchange in ^2H_2O. The effects of the two preexchange procedures are shown in Figure 1. Spectrum a is chymotrypsinogen A lyophilized three times from ^2H_2O. The region of the spectrum between 8 and 10 ppm contains a high intensity from numerous overlapping NH resonances. Preexchange either by heating in ^2H_2O (Figure 1b) or by unfolding in 6 M guanidinium chloride (Figure 1c) simplifies the aromatic region considerably. Intensity is removed both from the low-field region below δ 7.5 and from the aromatic envelope itself (δ 7.5–6.0). Both preexchange protocols permitted the resolution of two singlet low-field resonances labeled BCtg-H1 and BCtg-H2, which shift with pH. Further exchange was achieved by dissolving a heat-exchanged sample of chymotrypsinogen A in 2 M guanidinium hydrochloride in ^2H_2O (pH* 8.0) for 21 days at 25 °C. After desalting, the protein showed normal activity upon trypsin activation. A spectrum of the twice-exchanged zymogen is shown in Figure 1d.

We have determined that peaks H1 and H2 of chymotrypsin (Figures 2f-j), prepared from chymotrypsinogen A by trypsin activation at 25 °C, correspond to chymotrypsin A_α present in these samples. Chymotrypsin A_δ produced by rapid activation at 5 °C inexplicably does not yield resolved peaks in the histidyl region. The peaks appear after incubation of chymotrypsin A_δ samples at room temperature for a few hours at neutral pH which leads to the appearance of a second band on gels. On the other hand, chymotrypsin A_α preexchanged directly gives peaks identical with those of the activation product formed at 25 °C. In earlier reports, peaks H1 and H2 were attributed erroneously to chymotrypsin A_δ (Porubcan et al., 1975; Ibañez et al., 1976a,b).[3] All the results with preexchanged chymotrypsinogen A activated at 25°C have been verified directly using preexchanged chymotrypsin A_α.

The expected total intensity for the core of nonexchangeable aromatic resonances is 90 protons (2×2 His + 6×5 Phe + 4×4 Tyr + 8×5 Trp). The relative intensities of peaks BCtg-H1 and BCtg-H2 (Figure 1d) based on a total spectral intensity of 90 protons are 0.45 and 0.48 protons, respectively.

[3]Note Added in Proof: The identity of the species of chymotrypsin giving rise to peaks H1 and H2 was confirmed in early September, 1978.

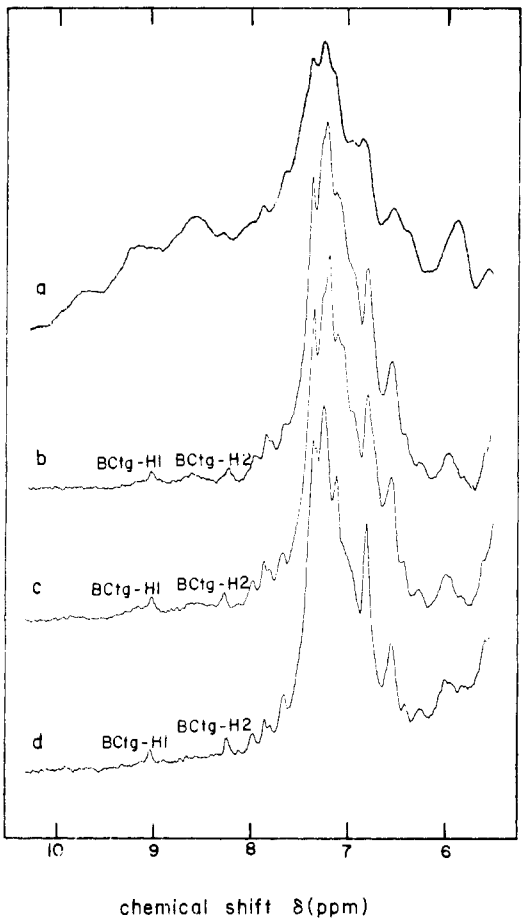


FIGURE 1: Comparison of 250-MHz correlation ^1H NMR spectra of bovine chymotrypsinogen A before (spectrum a) and after (spectra b–d) exchange of slowly exchangeable hydrogens in ^2H_2O. The exchange procedures (see text) simplify the histidyl $C^{\epsilon 1}$-H region of the spectrum and permit resolution of peaks BCtg-H1 and -H2 corresponding to individual histidyl residues. Sample conditions: 25 mg of protein/0.5 mL of 0.5 M KCl in ^2H_2O, pH* 3.0, 31 °C. (a) Sample lyophilized three times from ^2H_2O. (b) Sample exchanged in ^2H_2O at pH* 3.0 for 60 min at 60 °C. (c) Sample exchanged in ^2H_2O containing 6 M guanidinium chloride at pH* 3.0 for 12 h at 25 °C and then desalted. (d) Sample first exchanged as in spectrum b by heating in ^2H_2O, then incubated in 2 M guanidinium chloride in ^2H_2O at pH* 8.0 for 21 days at 25 °C, and finally desalted.

Chymotrypsin samples also yield integrated intensities of about 0.5 proton for peaks BCtr-H1 and H2. The fractional intensity of the His $C^{\epsilon 1}$-H peaks is disturbing. Since the singlet peaks have similar areas and normal line widths, it seems reasonable to assume that they represent single protons from the predominant form of native protein. We presume that their calculated fractional intensity indicates that this region contains an intensity from heterogeneous protein or that exchange of NH's under the aromatic envelope is incomplete. The same two peaks, also having fractional intensity, appear in spectra of preexchanged chymotrypsinogen and chymotrypsin that have been purified by chromatography on an insoluble BPTI column.

First-Stage Assignment of Histidyl Peaks. Peaks BCtg-H1 and H2 are assigned to the $C^{\epsilon 1}$ protons of the two histidyl residues of chymotrypsinogen by the following arguments: (1) The lack of exchange of the peaks indicates that they arise from CH groups rather than NH groups (Figure 1). (2) Histidine and tryptophan are the only amino acids that normally give rise to singlet CH peaks in this region. (3) The two peaks titrate with pH as expected for histidine (Figures 2 and 4). (4) The pH dependence of the chemical shifts is different for the two peaks (Figure 4), and only one peak is altered in DIP-BCtg (Figure 6) and $BCtr_\alpha$–BPTI (Figure 8). Therefore, the two peaks must correspond to different histidines in the sequence rather than either to the $C^{\delta 2}$-H and $C^{\epsilon 1}$-H of a single histidyl residue or to the same histidyl proton in different protein conformations that interconvert slowly on the NMR time scale. (5) Since both peaks exhibit abnormal histidyl titration curves, it is unlikely that either one corresponds to denatured protein. (6) The chemical shifts of the peaks are characteristic of $C^{\epsilon 1}$-H rather than $C^{\delta 2}$ resonances.

The slow rate of exchange of BCtg-H1 and -H2 at pH* 8 in 2 M guanidinium chloride in ^2H_2O implies that both histidines are still in their native environments. Preliminary experiments in 6 M guanidinium hydrochloride in ^2H_2O (I. B. Ibañez and J. L. Markley, unpublished) indicate that peaks BCtg-H1 and -H2 exchange more rapidly under more strongly denaturing conditions.

Similar arguments lead to assignments of the two peaks in ^1H NMR spectra of chymotrypsin A_α (Figure 2), which appear in the same region and titrate with pH (Figure 5), to the $C^{\epsilon 1}$ protons of His^{40} and His^{57}. The activation of chymotrypsinogen A has been followed in the NMR tube. Both peaks BCtr-H1 and -H2 appear immediately following activation by trypsin at 30 °C. Both peaks also are exhibited by commercial samples of chymotrypsin A_α.

Histidyl Titration Curves of Chymotrypsinogen and Chymotrypsin. ^1H NMR spectra of BCtg(pre-exch) and $BCtr_\alpha$(pre-exch) at various pH* values are compared in Figure 2. The line widths of the histidyl peaks of chymotrypsinogen A are considerably broader than those of chymotrypsin A_α (BCtr-H2). The line widths of the chymotrypsinogen A peaks become increasingly broader as the pH* is raised. The difference in line widths between zymogen and enzyme could be caused by differences in the state of aggregation (Tung and Steiner, 1974; Miller et al., 1971) which would affect the rotational correlation time. If one makes the simple assumption that the width of the histidyl peak is proportional to the molecular weight (Wüthrich et al., 1968), then, since the line widths of histidyl $C^{\epsilon 1}$-H peaks of ribonuclease (M_r 13 600) are typically 5 Hz at 250 MHz (Markley, 1975b), a line width of 9 Hz is expected for chymotrypsinogen or chymotrypsin (M_r 25 500). Alternatively, the excess line width in chymotrypsinogen A could be caused by exchange broadening from conformational changes occurring within the "disordered region" of the zymogen (Huber and Bode, 1977). Additional experiments are required to distinguish between these possibilities.

Resolution-enhancement techniques (Ernst, 1966) were used to follow peaks overlapping the aromatic envelope at high pH*. Examples of resolution-enhanced spectra are shown in Figure 3.

The chemical shifts of the peaks assigned to the histidyl residues of chymotrypsinogen A and chymotrypsin A_α are plotted as a function of pH* in Figures 4 and 5. The smooth curves are the result of least-squares analyses of the data; the titration parameters derived from these calculations are presented in Table I.

In chymotrypsinogen, the titration curve of peak BCtg-H1 (Figure 4) displays two transitions with pK' values of 7.3 and 1.4. The curve with pK' 7.3 is displaced 0.6-ppm downfield from a normal histidyl titration curve (based on the chemical shift of the protonated form). This indicates that the histidyl residue is located in an unusual deshielding environment. The process occurring with the low pK' (1.4) normalizes the chemical shift of the $C^{\epsilon 1}$ proton and must disrupt the unique environment of the histidyl ring. Peak BCtg-H2 also exhibits two pH inflections (Figure 4). Peak BCtg-H2 may be followed

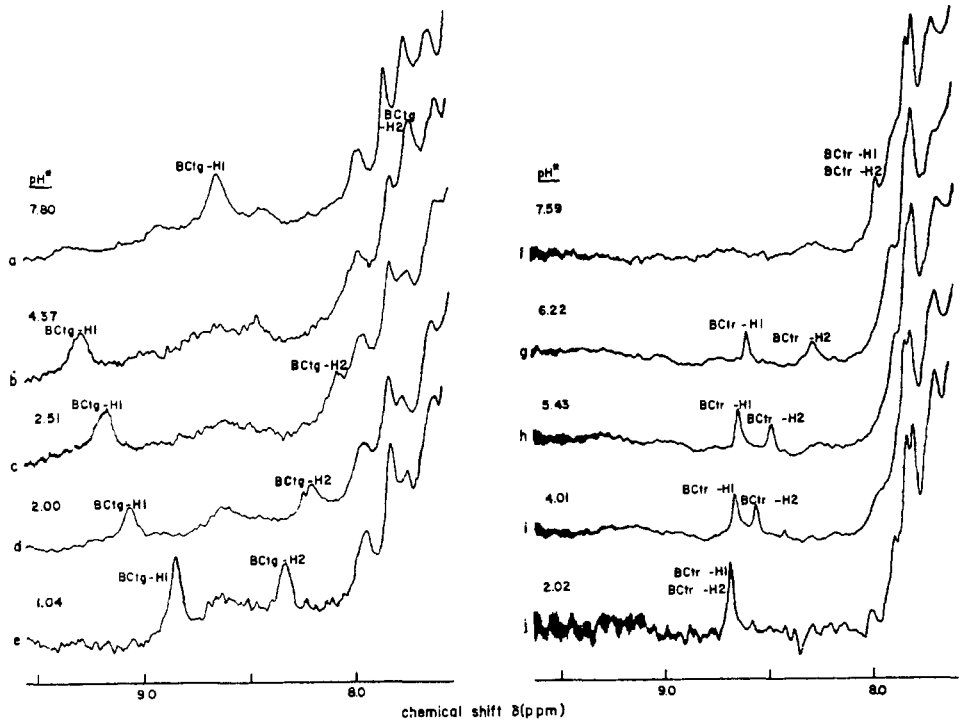


FIGURE 2: 250-MHz correlation ^1H NMR spectra of the histidyl $C^{\epsilon 1}$-H regions of heat preexchanged bovine chymotrypsinogen A (spectra a–e) and bovine chymotrypsin A_α prepared from the preexchanged zymogen (spectra f–j) at selected pH* values shown in the figure: 25 mg of protein/0.5 mL of 0.5 M KCl in ^2H_2O, 31 °C.

TABLE I: Transitions Affecting the Histidyl $C^{\epsilon 1}$-H NMR Peaks of Bovine Chymotrypsinogen A, Chymotrypsin A_α, and DIP-chymotrypsinogen A.[f]

His NMR peak	res. assign.	pK' value	Hill coeff[a]	NMR chem shift (ppm from DSS) $\delta_{low\ pH}$	$\delta_{high\ pH}$	$\Delta\delta_{low-high}$
BCtg-H1[b]	Asp^{102}	1.36 ± 0.03	c	8.65 ± 0.01	9.21 ± 0.03	−0.56 ± 0.04
	His^{57}	7.33 ± 0.02	c	9.21 ± 0.03	8.44 ± 0.01	0.77 ± 0.04
BCtg-H2[b]	Asp^{194} [d,e]	2.29 ± 0.06	c	8.35 ± 0.01	7.96 ± 0.01	0.39 ± 0.02
	His^{40}	4.57 ± 0.04	c	7.96 ± 0.01	7.42 ± 0.01	0.44 ± 0.02
BCtg-H1	Asp^{102}	1.06 ± 0.07	0.67 ± 0.04	8.49 ± 0.03	9.22 ± 0.03	−0.73 ± 0.04
	His^{57}	7.33 ± 0.02	0.96 ± 0.04	9.22 ± 0.01	8.44 ± 0.01	0.78 ± 0.02
BCtg-H2	Asp^{194} [d,e]	2.26 ± 0.05	1.08 ± 0.12	8.36 ± 0.01	7.96 ± 0.00	0.40 ± 0.01
	His^{40}	4.57 ± 0.02	0.87 ± 0.04	7.96 ± 0.00	7.51 ± 0.01	0.45 ± 0.01
$BCtr_\alpha$-H2[b]	Asp^{102} [d]	2.77 ± 0.25	c	8.69 ± 0.02	8.57 ± 0.01	0.12 ± 0.03
	His^{57}	6.14 ± 0.04	c	8.57 ± 0.01	7.95 ± 0.01	0.62 ± 0.02
$BCtr_\alpha$-H1	His^{40}	7.20 ± 0.03	0.90 ± 0.04	8.67 ± 0.01	7.69 ± 0.02	0.98 ± 0.03
$BCtr_\alpha$-H2	Asp^{102} [d]	2.91 ± 0.07	1.26 ± 0.26	8.68 ± 0.01	8.56 ± 0.01	0.12 ± 0.02
	His^{57}	6.18 ± 0.03	1.35 ± 0.13	8.54 ± 0.01	7.96 ± 0.01	0.58 ± 0.02
DIP-BCtg-H1[b]	Asp^{102}	3.14 ± 0.10	c	8.85 ± 0.01	9.06 ± 0.01	−0.21 ± 0.02
	His^{57}	7.63 ± 0.03	c	9.06 ± 0.01	8.44 ± 0.01	0.62 ± 0.02
DTP-BCtg-H2[b]	Asp^{194} [d,e]	2.14 ± 0.11	c	8.39 ± 0.03	7.99 ± 0.02	0.40 ± 0.05
	His^{40}	4.65 ± 0.08	c	7.99 ± 0.02	7.51 ± 0.01	0.48 ± 0.03

[a] A measure of cooperativity of the transition. [b] Two pK' values calculated simultaneously, assuming Hill coefficients of unity. [c] Not calculated. [d] In these cases, there may be some electron transfer from Asp^{102} to His^{57}; see eq 3 and 4 in the text. [e] This titration curve is fitted according to an alternative model in Table II and Figure 10. [f] The data were fitted using a nonlinear least-squares computer program.

only with difficulty because it moves into the envelope of aromatic peaks. In our earlier analysis (Porubcan et al., 1975) only the titration step at lower pH was detected, and an erroneous pK' was ascribed to the histidine. Further experiments, including the use of resolution-enhancement techniques (Figure 3), revealed that peak BCtg-H2 moves farther upfield as the pH is raised. The two inflection points occur at pH* 2.3 and 4.6.

In chymotrypsin A_α, peak BCtr-H1 (Figure 5) yields a normal titration curve with a single inflection with a pK' of 7.2. Peak BCtr-H2 is affected by two transitions having inflections at pH* 6.1 and 2.8. Above pH* 9, peak BCtr-H2 disappears, and a new peak appears at δ 8.4. This transition, which is slow on the NMR time scale, probably corresponds to the high pH inactivation investigated in detail by other methods (Oppenheimer et al., 1966; Hess et al., 1970; Fersht and Requena, 1971). A thorough ^1H NMR study of this transition will be described in a separate publication (Ibañez and Markley, in preparation). It is significant that the chemical shift of the low-field peak of the high-pH inactivation product corresponds exactly to that of peak BCtg-H1 of chymotrypsinogen. It has been proposed that this transition which is brought about by

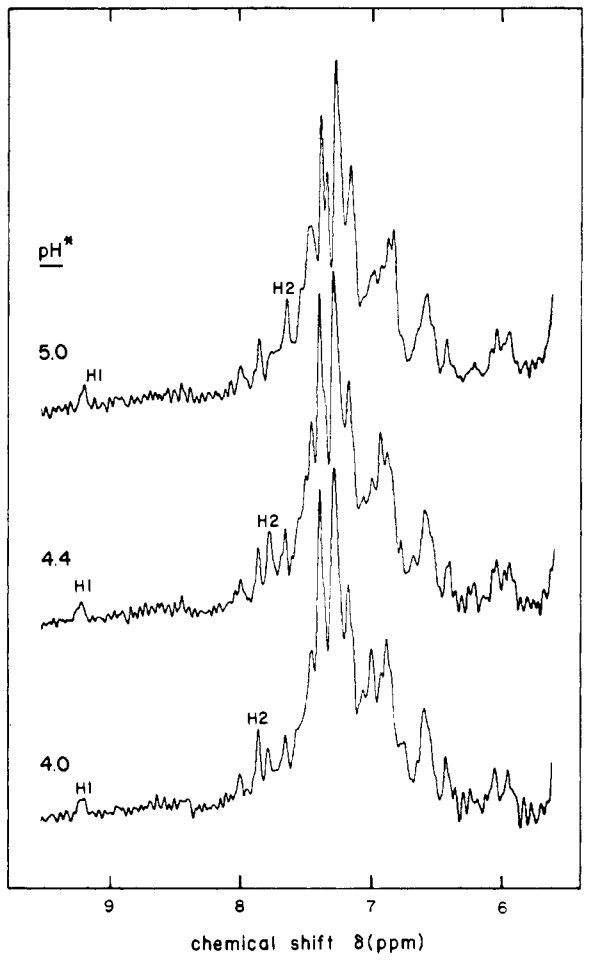


FIGURE 3: Examples of resolution enhancement (10.10.100) used to observe peak BCtg-H2 of chymotrypsinogen. Other conditions as in Figure 2. Spectra at three pH* values (indicated in the figure) are shown. His $C^{\varepsilon 1}$-H peaks are labeled H1 and H2.

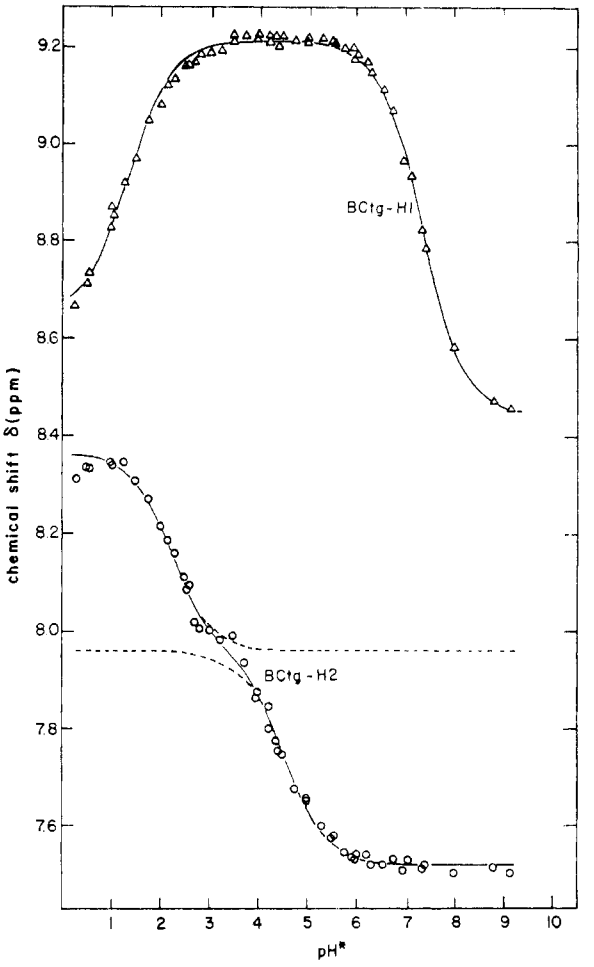


FIGURE 4: ^{1}H NMR titration curves (at 250 MHz) of the histidyl $C^{\varepsilon 1}$-H peaks of bovine chymotrypsinogen A, BCtg(pre-exch). Both peaks BCtg-H1 (Δ) and BCtg-H2 (○) are affected by two transitions. The assignments and pK' values are presented in Tables I and II. Sample spectra are shown in Figures 2 and 3.

deprotonation of Ile^{16} causes a reversion to a zymogen-like state (Oppenheimer et al., 1966).

DIP-chymotrypsinogen. Two ^{1}H NMR peaks appear in the histidyl $C^{\varepsilon 1}$-H region in spectra of DIP-BCtg(pre-exch). The chemical shifts of these peaks are compared with the histidyl titration curves of free bovine chymotrypsinogen A in Figure 6. The results indicate no significant change in the titration curve of peak BCtg-H2 after reaction with DFP. The other histidyl titration curve (BCtg-H1) is altered in the DIP derivative (DIP-BCtg-H1). The pK' of the lower transition is raised from 1.4 to 3.1, and the pK' of the higher transition is raised from 7.3 to 7.6. In addition, the chemical shifts of the low pH forms are altered (Table I).

DIP-chymotrypsin. Spectra of DIP-BCtr$_\delta$(pre-exch) demonstrate that one peak disappears, and the chemical shift of the other is altered. The peak that is not resolved may be missing because of exchange broadening. The titration curve of the resolved peak (Figure 7) exhibits a single inflection with a pK' of 7.3.

Chymotrypsin A_α–Pancreatic Trypsin Inhibitor Complex. The chemical shifts of the two low-field peaks visible in ^{1}H NMR spectra of the complex between bovine chymotrypsin A_α and bovine pancreatic trypsin inhibitor (Kunitz) are plotted as a function of pH in Figure 8. The dashed lines represent the titration curves of the histidines of uninhibited chymotrypsin A_α. A new peak, BCtr–BPTI-Hb, is observed in spectra of the complex which is not present in spectra of either component of the complex. Its chemical shift is constant at about 8.47 ppm

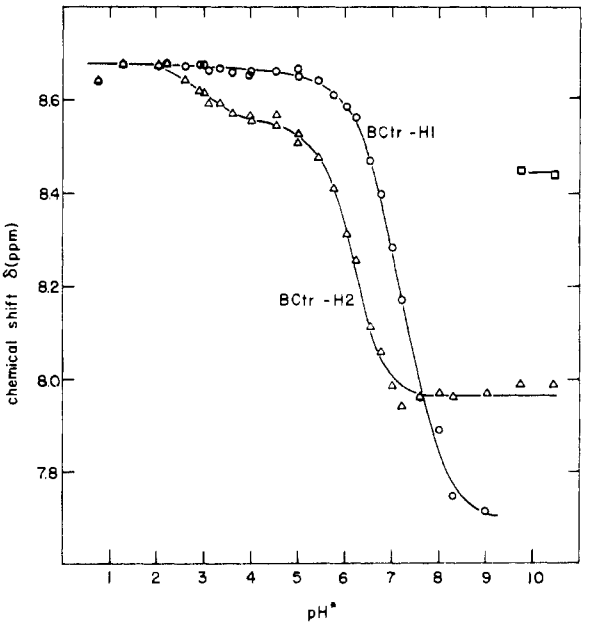


FIGURE 5: ^{1}H NMR titration curves (at 250 MHz) of the histidyl $C^{\varepsilon 1}$-H peaks of bovine chymotrypsin A_α, BCtr$_\alpha$(pre-exch). The assignments and pK' values are presented in Table I. Sample spectra are shown in Figure 2. Peak BCtr-H1 (○) is affected by a single transition. Peak BCtr-H2 (Δ) is affected by two transitions. A new peak (□) appears at above pH 9.5.

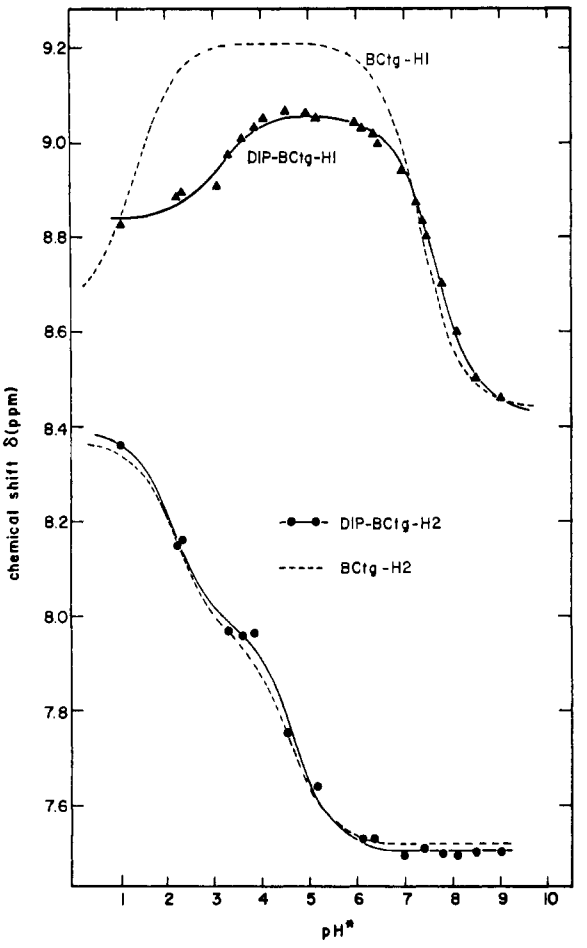


FIGURE 6: The pH dependence of the histidyl $C^{\epsilon 1}$-H ^{1}H NMR peaks (at 250 MHz) of bovine diisopropylphosphorylchymotrypsinogen A. Titration curves of free chymotrypsinogen A (- - -) are shown for comparison. Sample conditions: 25 mg of protein/0.5 mL of 0.5 M KCl in ^2H_2O, 31 °C.

between pH* 3.0 and 8.5. Below pH* 3.5 the peak disappears, and another peak, BCtr-BPTI-Hc, appears which follows the titration curve of BCtr-H_2. A third peak, BCtr-BPTI-Ha, follows the titration curve of BCtr-H1 from pH* 1-7. This peak could not be followed at higher pH because it shifts into the aromatic envelope and was no longer resolved. The broad aromatic envelope extends farther downfield in spectra of the complex than in spectra of free chymotrypsin or BPTI; hence, the histidine "window" becomes obscured. Convolution-difference techniques have not been useful in this case, apparently because the line width of the histidyl peak is comparable to those of overlapping aromatic peaks.

Discussion

The present NMR results provide the first direct spectroscopic data for both histidyl residues of chymotrypsinogen and chymotrypsin. The earlier NMR studies of Robillard and Shulman (1972, 1974a,b) concerned a single peak assigned to the proton hydrogen bonded between the His^{57} $N^{\delta 1}$ and Asp^{102} O^{δ} in chymotrypsinogen A and chymotrypsin A_{δ}. Data of this type are important for understanding mechanistic events because they provide the most reliable source of pK' values for proteins (Knowles, 1976) as well as information about the environments of individual groups in proteins. In order to interpret the present results, the two peaks observed in various samples and attributed to histidyl $C^{\epsilon 1}$ protons must be assigned to His^{40} or His^{57}, and the titration curves must be interpreted

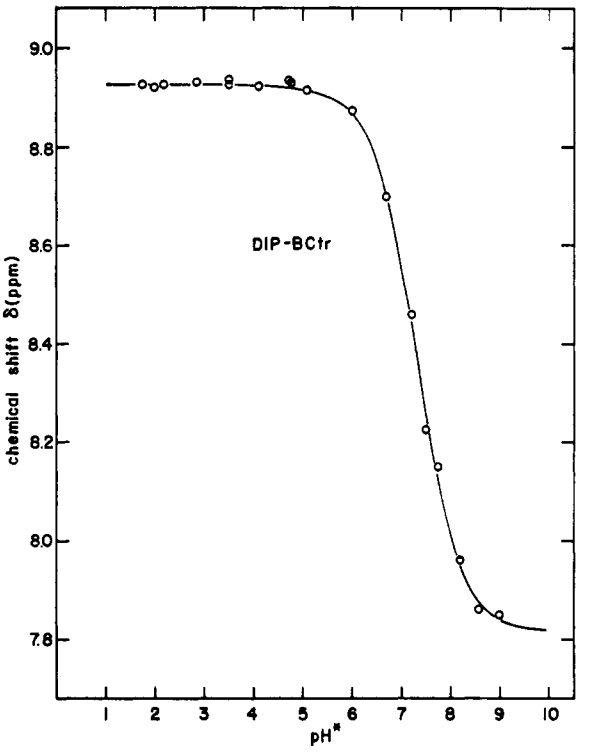


FIGURE 7: The pH dependence of the histidyl $C^{\epsilon 1}$-H ^{1}H NMR peak (at 250 MHz) of bovine diisopropylphosphorylchymotrypsin A_{δ}. Only one of the two histidyl peaks was resolved in the spectra. Sample conditions: 25 mg of protein/0.5 mL of KCl in ^2H_2O, 31 °C.

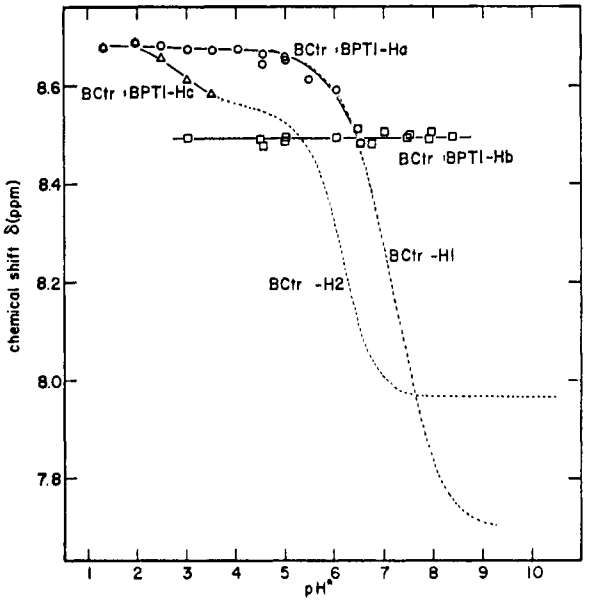


FIGURE 8: The pH dependence of the ^{1}H NMR peaks (at 250 MHz) assigned to the two histidyl residues of bovine chymotrypsin A_{α} in the complex with bovine pancreatic trypsin inhibitor. Titration curves of the histidyl $C^{\epsilon 1}$-H peaks in bovine chymotrypsin A_{α} alone (- -). Because of interfering peaks from the complex, peak BCtr-BPTI-Ha could not be traced at chemical shifts upfield of δ 8.4.

in terms of environmental effects and intrinsic and extrinsic protonation effects.

Assignments of the Histidyl $C^{\epsilon 1}$ H Peaks of Bovine Chymotrypsinogen A. We assign peak BCtg-H1 to His^{57} and peak BCtg-H2 to His^{40} according to the effects of diisopropylphosphorylation of Ser^{195} (Figure 6). It is expected that modification of Ser^{195} will have a greater effect on the adjacent

TABLE II: Comparison of the ^{1}H NMR Titration Behavior of Peaks Assigned to Histidine-57 $C^{\epsilon 1}$-H in Serine Proteinases and Serine Proteinase–Inhibitor Complexes.[f]

	$\delta_{high\ pH}$ (ppm from DSS)	1st protonation		2nd protonation	
		pK_1'	$\Delta\delta_1$	pK_2'	$\Delta\delta_2'$
BCtg	8.44	7.3	0.77	1.4	−0.56
BTg[a]	8.36	7.7	0.68	1.8	−0.43
PTg[a]	8.31	7.7	0.81	~3.4	−0.28
$BCtr_\alpha$	7.92	6.1	0.62	2.8	0.12
PTr[b]	7.94	5.0	0.41	4.5	0.28
α-lytic proteinase[c]	7.75	6.5	0.88	*c,d*	*c,d*
$BCtr_\alpha$–BPTI	8.47	3.0–3.5[g]		~2.8[g]	
PTr–BPTI[b]	8.30	~3.5[g]		~3.5[g]	

[a]Data from Porubcan et al. (1978). [b]Data from Markley and Porubcan (1976). [c]Data from Westler and Markley, unpublished. [d]The pK' of Asp^{102} is less than or equal to 4.2. [f]All data were obtained at 31 °C in ^2H_2O. [g]Cooperative; pH_{mid} values.

His^{57} to which it is hydrogen bonded (Birktoft et al., 1976) than on the more distant His^{40}. One peak (H1) is affected and one peak (H2) is not affected by reaction of chymotrypsinogen A with DFP. Furthermore, ^{31}P NMR studies of DIP-chymotrypsinogen A show that the chemical shift of the DIP phosphorus is influenced by two pH transitions having pK' values of 3.3 and 7.5 which match those of the peak assigned to His^{57} in the present study (Ibañez and Markley, to be published).

The unusual biphasic titration curve of BCtg-H1 appears to be characteristic of His^{57} in serine proteinase zymogens. Similar curves have been obtained with bovine and porcine trypsinogen (Porubcan et al., 1978; see Table II). In the case of the trypsinogens, the assignments to His^{57} are even more certain, since they were based on the ^{1}H NMR pH titration behavior of BPTI complexes (Porubcan et al., 1978) as well as ^{1}H and ^{31}P studies of DIP derivatives (Porubcan and Markley, to be published).

Assignments of the Histidyl $C^{\epsilon 1}$ H Peaks of Bovine Chymotrypsin A_α. We assign peak BCtr-H1 to His^{40} and peak BCtr-H2 to His^{57} based on the result of the BPTI binding experiment (Figure 8). The results parallel those obtained for the binding of BPTI to porcine trypsin (Markley and Porubcan, 1976) in which the peak assigned by independent means to the $C^{\epsilon 1}$ H of His^{57} of free trypsin has a pH-independent chemical shift in the complex (see Table II). The other three histidyl residues of porcine trypsin titrate normally in the complex. The X-ray structure of the bovine trypsin–BPTI complex (Rühlman et al., 1973) reveals that His^{57} lies in the contact region between the two proteins. Thus, it is expected that His^{57} will be perturbed as long as the normal structure of the complex remains intact. His^{40}, on the other hand, is not in contact with BPTI in the complex and appears not to be affected by complex formation. Peak BCtr–BPTI-Hb (Figure 8) appears only in spectra of the complex and has a pH-independent chemical shift slightly upfield from the position of the intermediate stage of curve BCtr-H2 of free chymotrypsin A_α. No NMR peak of the complex is observed to follow the curve BCtr-H2. Hence, peak BCtr-H2 is assigned to His^{57} in free BCtr, and peak BCtr–BPTI-Hb is assigned to His^{57} in the BCtr–BPTI complex. Peak BCtr–BPTI-Hc is also assigned to His^{57}. At low pH the complex undergoes a conformational transition (Engel et al., 1974) which allows protonation of the catalytic triad. In the transition region (at pH* 3), two peaks are observed, and their areas sum to roughly the area of peak BCtr–BPTI-Ha. Peaks BCtr-H1a,b,d and BCtr–BPTI-Ha are assigned by difference to His^{40} of free and complexed chymotrypsin A_α, respectively.

Support for this assignment comes also from studies of benzeneboronic acid inhibited chymotrypsin (I. B. Ibañez, W. M. Westler, and J. L. Markley, unpublished) in which the peak assigned to His^{40} is unperturbed whereas that assigned to His^{57} is perturbed.

The data for DIP-chymotrypsin A_δ are not useful for assignment purposes, since both histidines are perturbed by the chemical modification. The single resolved histidyl peak (Figure 7) probably corresponds to His^{40}, since it shows only a single inflection between pH 2 and 9. The ^{31}P NMR chemical shift of the phosphate of DIP-chymotrypsin A_α shows two inflections with pK' values of 4.3 and 7.5 (I. B. Ibañez and J. L. Markley, to be published). The absence of the second histidyl ^{1}H NMR peak could be the result of exchange broadening caused by a local conformational equilibrium in the His^{57}–Ser^{195}–DIP region of the molecule. Gorenstein and Findlay (1976) resolved two peaks in ^{31}P NMR spectra of DIP-chymotrypsin A_α which they attributed to two interconverting isomers of the same enzyme derivative. The interconversion rate was less than 1 s^{-1}, which appears to be too slow to explain the disappearance of the ^{1}H NMR peak.

Analysis of the NMR Titration Curves of His $C^{\epsilon 1}$ H's. The titration behavior of over 100 histidyl $C^{\epsilon 1}$ protons in over 30 different proteins has been investigated by ^{1}H NMR spectroscopy (for a review of older literature, see Markley, 1975a). A few generalizations emerge concerning ^{1}H NMR titration curves of histidines. The histidyl $C^{\epsilon 1}$ H of the model peptide Gly-His-Gly has a chemical shift of 7.70 ppm when the ring is uncharged and a chemical shift of 8.68 ppm when the ring is positively charged; the imidazole pK' is 6.8 ppm (Markley, 1975a). From studies of *N*-methylhistidines, the chemical shift of the $N^{\delta 1}$ tautomer is expected to be 0.11-ppm downfield from that of the $N^{\epsilon 2}$ tautomer (W. M. Westler and J. L. Markley, unpublished). Thus, in the absence of environmental shifts, the protonation shift of histidine in a peptide is expected to be 0.98 ppm for the $N^{\epsilon 2}$-H tautomer and 0.87 ppm for the $N^{\delta 1}$-H tautomer.

In proteins, the magnitude of the observed protonation shift may be larger or smaller than these values. For example, the titration shift for His^{12} of bovine pancreatic ribonuclease A is 1.37 ppm (Markley, 1975b) instead of the value of 0.87 ppm expected for the $N^{\delta 1}$ tautomer. Deviations from the normal titration shift result from preferential shielding or deshielding of one of the protonation states of the imidazole. The potential size of environmental shifts may be appreciated by examining the range of chemical shifts found for His $C^{\epsilon 1}$ protons in nonmetallo proteins. Previous to the present study, the maximum deshielding observed was 0.41 ppm for the protonated form of His^{12} of bovine pancreatic ribonuclease, and the maximum

TABLE III: Comparison of the ^1H NMR pH Titration Data for Histidine-57 $C^{\epsilon 1}$-H of Chymotrypsin A_α with Two Models for the Protonation States of the Charge Relay.

	model A: $pK_{His^{57}} > pK_{Asp^{102}}$			model B: $pK_{His^{57}} < pK_{Asp^{102}}$		
	E^- [c]	$EH^{+,-}$ [c]	HEH^+ [c]	E^- [c]	HE [c]	HEH^+ [c]
predict. chem shift (δ_{pred}) [a]	7.81	8.68	8.68	7.81	7.70	8.68
obsd chem shift (δ_{obsd}) [b]	7.95	8.57	8.69	7.95	8.57	8.69
environ shift ($\delta_{obsd} - \delta_{pred}$)	0.14	−0.11	0.01	0.14	0.87	0.01
predict. His protonat shift:	$(\delta_{EH^{+,-}} - \delta_{E^-})_{pred} = 0.87$			$(\delta_{HEH^+} - \delta_{HE})_{pred} = 0.98$		
obsd His protonat shift:	$(\delta_{HEH^{+,-}} - \delta_{E^-})_{obsd} = 0.62$			$(\delta_{HEH^+} - \delta_{HE})_{obsd} = 0.12$		

[a]Chemical shifts predicted from model studies (Markley, 1975a; W. M. Westler and J. L. Markley, unpublished). [b]Computer-fitted values for the plateaus of the titration curve (from Table I). [c]The structures of the species are defined in Figure 10.

shielding was −0.45 ppm observed with the protonated form of His^{48} of the same enzyme (Markley, 1975b). Environmental shifts larger than this can be envisioned if the histidyl $C^{\epsilon 1}$ H is located in the anisotropic shielding or deshielding regions of an aromatic ring. Environmental shifts also may be pH dependent. The magnitudes of pH-dependent environmental shifts are usually small but may approach that of the protonation of histidine itself. In the absence of additional data, it may not be possible to distinguish an intrinsic titration shift resulting from protonation of the histidine itself from an extrinsic titration shift whereby the environment of the histidine is affected by protonation of another residue.

Titration of His^{57} in Chymotrypsinogen A. Curve BCtg-His^{57} (Figure 9) exhibits two inflections having opposite first derivatives. These are the only inflections observed between pH* 0.5 and 9.5. Both Asp^{102} and His^{57} are expected to titrate in this range, and protonation of each is expected to influence the chemical shift of the His $C^{\epsilon 1}$ H. The positive inflection with $pK' = 1.4$ has a magnitude of −0.56 ppm and can be assigned unambiguously to an environmental perturbation. It probably corresponds to the pK' of Asp^{102} which is hydrogen bonded to His^{57}. In the following paper (Porubcan et al., 1978), a mechanism is suggested for the deshielding of peak BCtg-H1 based on the X-ray structure of trypsinogen (Bode et al., 1976). The deshielding interaction apparently is broken up at low pH as a consequence of protonation of Asp^{102} or some other carboxylate. The negative inflection with $pK' = 7.3$ is assigned to the protonation of His^{57}. The magnitude of the shift (0.77 ppm) is close to the value predicted for protonation of the $N^{\delta 1}$-H histidyl tautomer (0.87 ppm).

Titration of His^{57} in Chymotrypsin A_α. Curve BCtr-His^{57} (Figure 9) exhibits two inflections, both of which have negative first derivatives. Since only minor structural changes in the region of His^{57} between pH 4.2 and 6.7 (Vandlen and Tulinsky, 1973) are observed by X-ray crystallography, we assume that the two observed titration steps correspond to His^{57} itself and the adjacent Asp^{102}. Here it is more difficult to assign which inflection corresponds to the titration of His^{57} and which corresponds to an environmental perturbation by Asp^{102}. The two possible cases are compared in Table III. We find it more reasonable to assign the higher pK' to His^{57} and the lower pK' to Asp^{102} (model A, Table III) than the reverse for the following reasons. (1) The shift of 0.62 ppm is closer to the value of 0.87 ppm expected for protonation of the $N^{\delta 1}$-H histidyl tautomer than the shift of 0.12 ppm. (2) If the opposite assignment were made (model B, Table II) one would have to invoke a uniquely large environmental shift for the "neutral" histidine (0.87 ppm at pH* 4). Examination of available X-ray structures (Vandlen and Tulinsky, 1973) does not provide any basis for such a large environmental shift. (3) Similar ^1H NMR titration curves having reduced protonation shifts have been reported for His^{57} of porcine trypsin (0.41 ppm; Markley and Porubcan, 1976), His^{48} of ribonuclease A (0.59 ppm; Markley, 1975b), and His^{71} of soybean trypsin inhibitor (Kunitz) (0.57 ppm; Markley, 1973; Markley and Kato, 1975). In all cases, the small protonation shift results from shielding of the positively charged imidazole, as is proposed here for chymotrypsin.

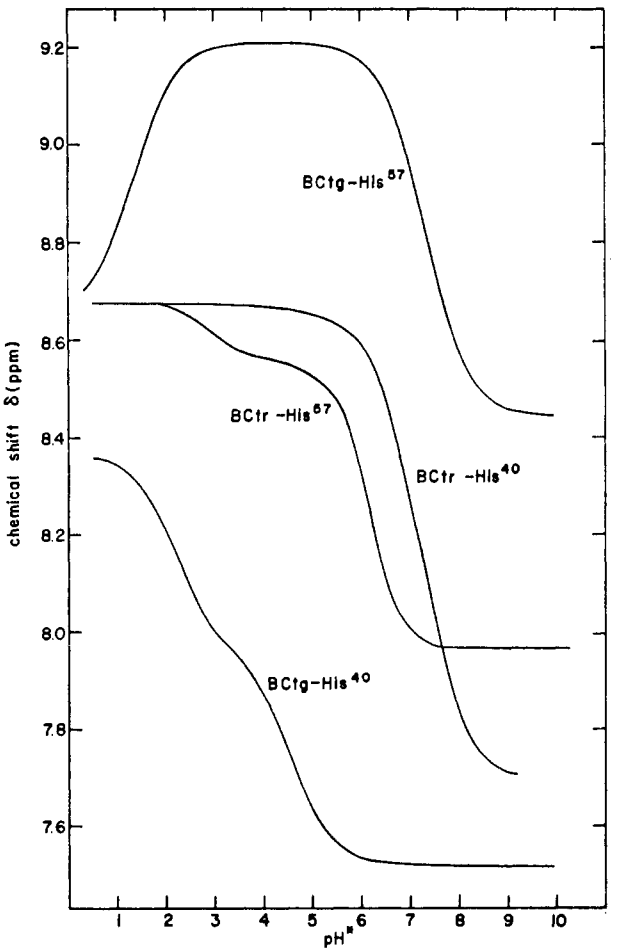
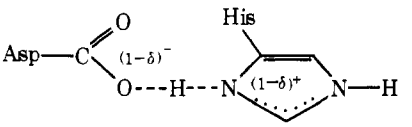


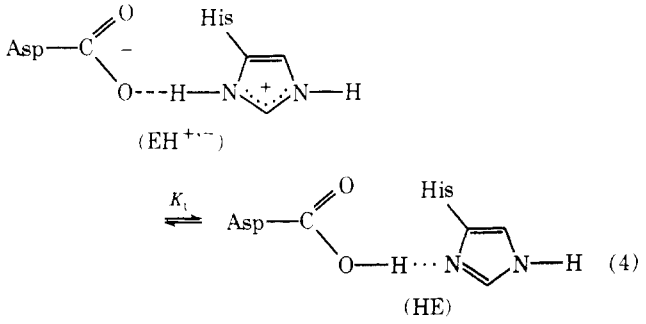
FIGURE 9: Comparison of the ^1H NMR titration curves (at 250 MHz) assigned to the $C^{\epsilon 1}$-H of histidine-40 and -57 of bovine chymotrypsinogen A (BCtg) and bovine chymotrypsin A_α (BCtr).

Shielding of the His $C^{\epsilon 1}$ H could arise from partial neutralization of the positive charge on the histidyl ring by a carboxylate according to one of two mechanisms: (1) partial sharing of the histidyl $N^{\delta 1}$ H with the aspartate resulting from lengthening of the hydrogen bond (eq 3); (2) full proton



(3)

transfer resulting in a tautomeric equilibrium (eq 4).



(4)

In the latter case, the chemical shift of the singly protonated Asp-His would be the weighted average of the chemical shifts of the two species

$$\delta = \delta_{EH^{+,-}}[EH^{+,-}] + \delta_{HE}[HE]/[EH^{+,-}] + [HE] \quad (5)$$

The single energy minimum (eq 3) or the double energy minimum (eq 4) models cannot be distinguished on the basis of the present data. If environmental effects are constant throughout the titration of His^{57} (or can be evaluated), the NMR results provide a quantitative measure of the degree of charge delocalization. There may be reason to believe that the extent of charge delocalization varies among different active serine proteinases related to chymotrypsin, since the magnitudes of the protonation shifts observed for porcine trypsin (0.41 ppm) (Markley and Porubcan, 1976), bovine chymotrypsin A_α (0.62 ppm), and α-lytic proteinase (0.88 ppm) (Westler and Markley, 1978) are significantly different (Table II). Other environmental factors, however, could be responsible for these differences.

Titration of His^{40} in Chymotrypsinogen A. Curve BCtg-His^{40} (Figure 4) deviates significantly from a single titration curve. A large number of data points was collected in the pH region 2–5, where the peak is easily resolved. The observation of a shielded, biphasic titration curve for His^{40} is consistent with the X-ray structure of chymotrypsinogen (Freer et al., 1970), which indicates that His^{40} is located in a hydrophobic region and that there is an interaction between the imidazole of His^{40} and the carboxylate of Asp^{194}.

Curve BCtg-H2 (BCtg-His^{40}) has been analyzed in the same way used for curve BCtr-H2 (BCtr-His^{57}). Two pK' values of 2.29 and 4.57 are obtained with titration shifts, respectively, of 0.39 and 0.44 ppm. The larger step is assigned tentatively to the titration of His^{40} and the smaller step to the titration of Asp^{194}. The abnormally small titration shift may be explained by charge delocalization, as shown in eq 3 or 4. When Asp^{194} becomes protonated, the normal chemical shift of His^{40} is realized. The titration curve shown in Figure 4 represents a "best fit" according to this model.

Titration of His^{40} in Chymotrypsin A_α. Curve BCtr-His^{40} (Figure 9) is characteristic of a "normal" histidyl residue. It is fitted with a single pK' of 7.2 and a Hill coefficient of unity (Table I). The magnitude of the titration shift is a normal 0.98 ppm, and the chemical shifts of both the neutral and positively charged forms of the ring are those of a free histidyl side chain. The crystallographic structure of tosylchymotrypsin A_α obtained at pH 4.2 shows a hydrogen bond between the $N^{\delta 1}$ of His^{40} and the side chain O^γ of Ser^{32} (2.67 Å) and a hydrogen bond between the $N^{\epsilon 2}$ of His^{40} and the carbonyl of Gly^{193}. The $N^{\epsilon 2}$ also interacts with a water molecule, indicating that it is accessible to solvent (Birktoft et al., 1969; Birktoft and Blow, 1972). In the crystallographic structure of chymotrypsin A_α at pH 3.6, no hydrogen bonds are reported for the His^{40} ring (Tulinsky et al., 1973a,b). Tulinsky and co-workers found a change in the orientation of His^{40} in the difference electron-density maps between crystals of native enzyme at pH 6.7 and 4.2. At the higher pH, the imidazole ring of His^{40} rotates around in its plane toward the carbonyl of Gly^{193}. This change was attributed to deprotonation of His^{40} (Vandlen and Tulinsky, 1973). According to the present data, His^{40} should be only 24% deprotonated at pH 6.7. However, the pK' of His^{40} may be lowered by dimer contacts or crystal packing. Since His^{40} has a normal chemical shift at low pH, the NMR data provide no support for a charge-transfer complex proposed between His^{40} and Trp^{141} at low pH (Vandlen and Tulinsky, 1973).

State of the Charge Relay in the BCtr$_\alpha$-BPTI Complex. Results for the BPTI complexes of trypsin and chymotrypsin are compared in Table II. The chemical shift of the His^{57} $C^{\epsilon 1}$ H is similar in the two complexes, and the chemical shift is pH independent in the pH range where the complexes are most stable. The low pH transitions observed by ^1H NMR spectroscopy are compatible with the known pH dependences of the equilibrium constants of these complexes (Finkenstadt et al., 1974; Engel et al., 1974). We have examined the X-ray coordinates for the trypsin–BPTI complex and do not see any structural features that would account for the deshielding observed on complex formation at neutral pH. If this is also true for the chymotrypsin–BPTI complex, the chemical shifts indicate that His^{57} is positively charged in both complexes.

Summary of the Present NMR Results. The NMR studies from this laboratory lead to the following general conclusions: (1) The environment of His^{57} is very different in zymogens and activated enzymes. Both the pK' value of His^{57} and its mode of interaction with other active-site components are perturbed upon activation. This conclusion is independent of any assignments. (2) The first proton added to the active form of the Asp-His pair resides primarily on His^{57}. Hence, in both zymogens and enzymes the pK' of His^{57} is higher than the pK' of Asp^{102}. This conclusion depends on the correct assignment of the histidyl peaks and on the proper assignment of the chemical-shift changes to individual protonation steps. These assignments are more reliable in the case of chymotrypsinogen than of chymotrypsin as discussed above. (3) Since the pK' values of His^{57} and Asp^{102} in the free proteins appear to bear no direct relationship to the pK' values derived from steady-state kinetics studies of pH-rate profiles (Fersht, 1977), the simple mechanism proposed for catalysis may be inadequate. The pK' values observed in the steady state may reflect a combination of protonation steps or a single protonation of a residue removed from the active site (Jencks, 1969). (4) The pK' values of the active-site residues are perturbed readily by changes occurring at or near the active site. (5) His^{57} of chymotrypsin, trypsin (Markley and Porubcan, 1976), and trypsinogen (Porubcan et al., 1978) does not titrate when these proteins are in stable complexes with BPTI. This result depends on the correct assignment of the $C^{\epsilon 1}$-H peak of His^{57}. (6) His^{57} appears to have a positive charge in complexes between chymotrypsin or trypsin and BPTI. This conclusion assumes the correct assignment and that we have taken into account all large deshielding effects on the $C^{\epsilon 1}$ H of His^{57} that exist in the complexes (Porubcan et al., 1978).

Comparison of the Present Results with Previous Studies. The protonation states of the catalytic groups are summarized in Figure 10. In the zymogens, there is little evidence for the existence of species (HE). In the enzymes, K_1 may be slightly significant, since it could explain the abnormally small protonation shift of the His^{57} $C^{\epsilon 1}$ H; however, the data can be accommodated equally well by a sharing of the hydrogen-bonded proton of His^{57} with Asp^{102} in species $(EH^{+,-})$ (see

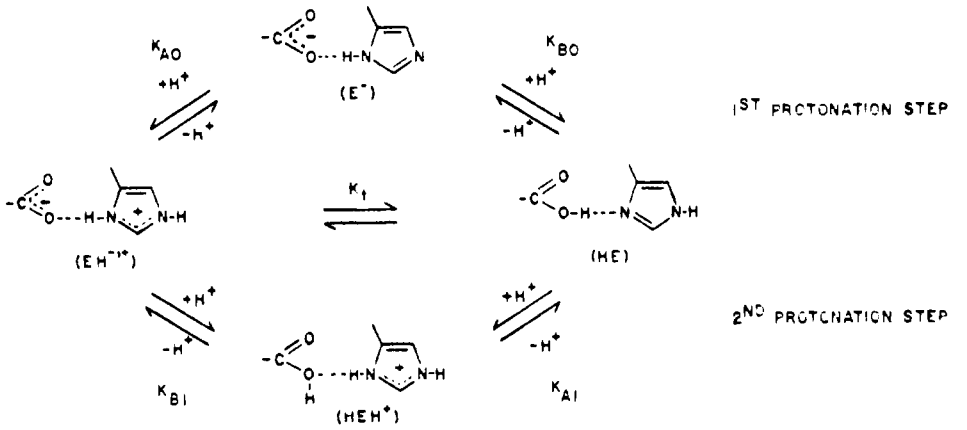


FIGURE 10: Protonation scheme for the charge-relay residues histidine-57 and aspartate-102 of serine proteinases.

eq 3) or by some other environmental effect. The NMR results give values for the first and second protonation steps shown in Table II. The pK' of His^{57} derived from NMR results is highly variable among enzymes and zymogens, whereas pK' values derived from steady-state kinetics generally have values in the narrow region of 6.8–7.2 (Hess, 1971; Gertler et al., 1974). Knowles (1976) has recently discussed the reliability of various techniques for the determination of intrinsic pK values of groups in enzymes. Steady-state kinetics have severe limitations and are judged to be the least reliable procedure in current use. More reliable are studies of the pH dependence of modification reactions, and most reliable are direct observations of pK' values by thermodynamic or spectroscopic methods.

The abnormally low pK' values assigned to Asp^{194} and Asp^{102} of chymotrypsinogen A and Asp^{102} of chymotrypsin A_α are consistent with the predictions of Fersht and Sperling (1973) that these groups have pK' values below 4.2. A subsequent kinetics study (Fersht and Renard, 1974) indicated that the pK' of the second protonation step of the catalytic triad is lower than 2. The present results yield a value slightly higher than this.

Cruickshank and Kaplan (1974) have derived pK' values for His^{40} and His^{57} of chymotrypsinogen and chymotrypsin by measuring the pH dependence of the reaction of these residues with 1-fluoro-2,4-dinitrobenzene. The pK' values they obtained were *chymotrypsinogen A:* His^{40}, 8.0; His^{57}, 7.5; and *chymotrypsin* A_α: His^{40}, 6.8; His^{57}, 7.0. These values are in relatively good agreement with our NMR results except for the pK' of His^{40} of chymotrypsinogen A (8.0) for which we obtain a pK' of 4.6. The discrepancy may indicate a pH dependence in the reaction mechanism with 1-fluoro-2,4-dinitrobenzene or a pH-dependent change in the accessibility of His^{40} of chymotrypsinogen A. The rate of inactivation of chymotrypsinogen by DFP is dependent on the ionization of a single group with a pK' of 7.15 (Gertler et al., 1974). This group presumably is His^{57} to which the present results assign a pK' of 7.3. These results suggest that a deprotonated His^{57} is still required in the zymogen for activity.

The pK' of His^{57} of chymotrypsin obtained in the present study is also in relatively good agreement with earlier thermodynamic results. Based on the pH dependence of the aggregation of chymotrypsin A_α, Aune and Timasheff (1971) ascribed pK' values of 5.0 and 6.2 to His^{57} in monomeric and dimeric chymotrypsin A_α. Their general experimental results have been confirmed more recently by Horbett and Teller (1974). Hanai (1976) recently obtained thermodynamic constants for the ionization of a group in the active site of chymotrypsin A_δ having a pK' value of 6.8 at 25 °C. These results were obtained through an analysis of proton release accompanying binding of the inhibitor, benzeneboronic acid. He assigned these to His^{57} because the ΔH value (6.8 kcal mol^{-1}) and ΔS value (0.8 cal mol^{-1} deg^{-1}) agreed favorably with ionization of imidazole in water (ΔH = 8.8 kcal mol^{-1}; ΔS = −2.8 cal mol^{-1} deg^{-1}) and not with ionization of carboxylic acids (ΔH = −0.02 kcal mol^{-1}; ΔS = −21.9 cal mol^{-1} deg^{-1}). It can be argued, however, that an abnormal group such as His^{57} may not have normal thermodynamic values. Similar pK' values (6.1–6.5) were obtained earlier by proton release studies of substrate binding to chymotrypsin (Fersht and Renard, 1974).

The present results are in partial agreement with the ^{1}H NMR investigations of Robillard and Shulman (1972, 1974a,b) on the pH dependence of the peak farthest downfield in ^1H_2O solutions of chymotrypsin A_δ and chymotrypsinogen A. They obtained pK' values near 7 for His^{57} in both enzyme and zymogen and also found that His^{57} does not titrate in the BCtr$_\delta$–BPTI complex. Robillard and Shulman did not extend their titrations below pH 3 and thus did not observe the second transition which affects His^{57}. Their conclusions (Robillard and Shulman, 1974a,b) differ from ours in three respects. (1) The NH assigned to His^{57} had the same chemical shift in both the enzyme and zymogen, except for a small inflection at pH 5.7 in the enzyme; therefore, it was argued that the catalytic triad has a similar structure in the zymogen and enzyme. (2) The same pK' (7.5) was obtained for His^{57} in enzyme and zymogen. (3) The chemical shift of the NH in the complex with BPTI was similar to that of the NH in chymotrypsin at pH 8; therefore, it was postulated that the catalytic triad does not become protonated upon complex formation. If the assignments of Robillard and Shulman are correct and there is no clear evidence to the contrary (Markley, 1978), the differences may be explained as follows: (1) The deshielding of His^{57} C$^{\epsilon 1}$ H in chymotrypsinogen but not chymotrypsin is attributed to a local anisotropy (Porubcan et al., 1978). The environment of His^{57} N$^{\delta 1}$ H may be equivalent in both zymogen and enzyme (Birktoft et al., 1976). (2) The discrepancy in pK' values between the present study and that of Robillard and Shulman (1974a,b) may be the result of a large difference in the ΔH of ionization of His^{57} in chymotrypsinogen and chymotrypsin or a large solvent deuterium isotope effect on the pK' of His^{57} in chymotrypsinogen. Robillard and Shulman's titration studies were carried out at 3 °C in ^1H_2O. Using the thermodynamic results of Hanai (1976) in ^1H_2O, a pK' of 7.2 is expected at 3 °C and a pK' of 6.6 is expected at 31 °C. These values are in reasonable agreement with Robillard and Shulman's pK' of 7.5 at 3 °C in ^1H_2O and the present pK' of 6.1 at 31 °C in ^2H_2O. Unfortunately, thermodynamic parameters are not at hand for chymotrypsinogen. (3) Recent studies of the low-field

NH of porcine trypsin (Markley, 1978) indicate that its chemical shift may be as responsive to environmental changes as to the charge state of His^{57}. In this case, the relationship between the chemical shift of the NH in complexes and the charge state of His^{57} may be more complicated than originally thought.

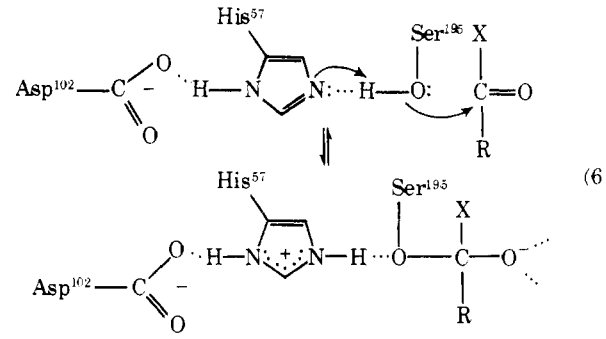
The results from this laboratory are not in agreement with those studies of serine proteinases that have attributed a low pK' (4.0 or below) to His^{57} (Hunkapiller et al., 1973) and a high pK' (6.8) to Asp^{102} (Hunkapiller et al., 1973; Koeppe and Stroud, 1976). Hunkapiller and colleagues (1973) carried out an elegant ^{13}C NMR study of α-lytic proteinase that had been selectively enriched with ^{13}C in the $C^{\epsilon 1}$ of the single histidyl residue which is homologous to His^{57} of chymotrypsin. As in the present study, the probe indicated that His^{57} is affected by two protonation steps. The first with pK' 6.8 altered the chemical shift of the $^{13}C^{\epsilon 1}$; the second with a pH_{mid} around 3.3 affected the chemical shift of the $^{13}C^{\epsilon 1}$ and its $^1J_{CH}$ coupling constant with the imidazole $C^{\epsilon 1}$-^1H. The latter transition involved a step that is slow on the NMR time scale. Hunkapiller et al. assigned the pK' around 3 to protonation of His^{57} and the pK' of 6.8 to the protonation of Asp^{102} on the basis of model studies, which indicated that the $^1J_{CH}$ coupling constant is a reliable indicator of the charge on the imidazole. In terms of Figure 10, the results were interpreted to mean that the first protonation step leads to species (HE) exclusively. The interpretation of Hunkapiller et al. may be incorrect if for some reason $^1J_{CH}$ is not responsive to the charge state of His^{57} in the enzyme or if the measurement was subject to error. The change in $^1J_{CH}$ observed between pH 5.2 and 3.3 was only 13 Hz, whereas the line widths of the peaks between which the coupling constant was measured were 28–32 Hz. (In contrast, in the present ^1H NMR study of chymotrypsin A_α the magnitude of the titration shift assigned to His^{57} is 13 times the width of the His^{57} peak.) It may be that under these conditions the chemical shift of His^{57} $C^{\epsilon 1}$ is a better measure of its charge state than the coupling constant (Robillard and Shulman, 1974a; Egan et al., 1977).

In the study by Koeppe and Stroud (1976), all but 2.5 carboxyls of bovine trypsin were blocked with semicarbazide. Both Asp^{102} and Asp^{194} were shown to be unmodified. Difference IR measurements of the stretching modes at 1570 and 1710 cm^{-1} were carried out as a function of pH. The results indicated that 1.5 carboxyls titrate with an average pK' of 2.9, while 1 titrates with a pK' of 6.8. The carboxyl with pK' 6.8 was assigned to Asp^{102}, since the high pH difference spectrum was abolished in the presence of 6 mM $CuCl_2$; Cu^{2+} was shown to bind specifically between Asp^{102} and His^{57} in DIP trypsin. This interpretation of the results requires (1) the absence of pH-dependent spectroscopic perturbations of the intense amide background in which the carboxyl bands are found and (2) no modulation of carboxyl stretching bands by adjacent titrating groups. These possibilities could not be ruled out completely. The ^1H NMR titration curve assigned to the $His^{57}C^{\epsilon 1}H$ of porcine trypsin (Markley and Porubcan, 1976) showed no inflection in the region around 6.8. In this study, Asp^{102} was assigned a pH_{mid} of 4.5.

The notion that the uncharged species (HE) is of lower energy than the ion pair ($HE^{+,-}$) has received support from recent molecular orbital calculations modeling the charge relay (Amidon, 1974; Scheiner et al., 1975; Kitayama and Fukutome, 1976; Beppu and Yomosa, 1977). These calculations have neglected dipoles from the surrounding protein and solvent which would stabilize the zwitterion ($HE^{+,-}$).

Other experimental results appear to favor species ($HE^{+,-}$) over species (EH) of Figure 10. A synthetic charge-relay model (Rogers and Bruice, 1974), incorporating adjacent imidazole and carboxylate groups which exhibited some catalytic activity, was found to have a significantly higher imidazole pK' than carboxylate pK' even in 96% dioxane. Crystallographic data concerning anion binding at the active site of serine proteinases also are indicative of a positive charge on His^{57} at low pH. Phosphate binds in the active-site region of *Streptomyces griseus* proteinase B at pH 4.2 (Delbaere et al., 1975). Sulfate apparently binds at the active site of one of the molecules of chymotrypsin A_α at pH 4 (Tulinsky et al., 1973a) and to the active site of elastase (Shotton et al., 1971) and trypsin (Huber and Bode, 1977) at pH 5 but not at pH 8.

Based on the above arguments we favor the following mechanism, over that given in eq 1, for formation of the tetrahedral intermediate:



Changes in the Catalytic Groups upon Zymogen Activation. There is a 10^6-fold increase in activity when chymotrypsinogen is activated as the result of cleavage of the Arg^{15}–Ile^{16} bond. The new amino terminal (Ile^{16}) forms an ion pair with Asp^{194} which clamps together a new hydrogen-bonding network. One view of zymogen activation, based on studies of bovine chymotrypsinogen and trypsin, is that the zymogen has a rigid structure with, however, a poorly developed binding pocket (Kraut, 1971; Wright, 1973a; Kossiakoff et al., 1977). An alternate view, based on studies of bovine trypsinogen, is that the zymogen contains a disordered region or "activation domain" which becomes rigid upon activation; the ordering of the domain is linked to the formation of the specificity pocket (Huber and Bode, 1977). The generation of substrate specificity upon activation has been substantiated by chemical studies which show that, of the 10^6-fold difference in enzyme/zymogen activity, 10^4 is attributable to differences in the binding site and 10^2 due to differences in the catalytic activity (Gertler et al., 1974). Differences in the structure and properties of the Asp-His-Ser triad revealed by the present work (Figure 9, Table II) and by recent refinement of the X-ray structures (Matthews et al., 1977; Birktoft et al., 1976) may account for part of the missing factor of 10^2. NMR studies of porcine and bovine trypsinogen (Porubcan et al., 1978) and porcine chymotrypsinogen A (Ibañez and Markley, unpublished) disclose a remarkable uniformity in the unusual properties of His^{57} in zymogens. The serine proteinases apparently have evolved a particular way of deactivating the catalytic groups. Any model of zymogen activation must take into consideration changes in the pK' values and environments of the Asp-His pair (Figure 9, Table II).

Another significant factor in the generation of catalytic activity upon activation is the structuring of the oxyanion hole (Robertus et al., 1972), as revealed by comparisons of the X-ray structures of zymogens and enzymes (Freer et al., 1970; Wright, 1973b; Fehlhammer et al., 1977; Kossiakoff et al., 1977). Recent ^{31}P NMR studies (Reeck et al., 1977) reveal a difference in the environment of the alkyl phosphate in DIP-chymotrypsinogen and DIP-chymotrypsin. Similar ^{31}P

NMR studies of DIP-trypsinogen and DIP-trypsin confirm this change (M. A. Porubcan and J. L. Markley, to be published).

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Active Site in Zymogens. Proton Magnetic Resonance pH Titration Curves of Histidine-57 in Porcine and Bovine Trypsinogens and in Their Complexes with Bovine Pancreatic Trypsin Inhibitor (Kunitz)†

Michael A. Porubcan, Darrow E. Neves, Steven K. Rausch, and John L. Markley*,‡

ABSTRACT: Peaks attributed to the $C^{\epsilon 1}$ protons of the four histidines of porcine trypsinogen have been resolved in 250-MHz ^1H NMR spectra after preexchange of overlapping N-H resonances in ^2H_2O at elevated temperature. One of the histidyl peaks is shifted abnormally far downfield at high pH and is affected by two transitions occurring with pH_{mid} values of 3.2 and 7.7. A similar peak observed in spectra of preexchanged bovine trypsinogen undergoes transitions with pH_{mid} values of 1.7 and 7.7. These peaks have been assigned to the $C^{\epsilon 1}$-H of histidine-57 (chymotrypsinogen numbering system) in the active site. The assignments are based on the perturbation of these peaks in diisopropylphosphoryl derivatives of bovine and porcine trypsinogen. The pK' values of 7.7 are assigned to protonation of histidine-57, and the pK' values of 3.2 and 1.7 are assigned to protonation of aspartate-102 in porcine and bovine trypsinogen, respectively. The titration behavior of histidine-57 in both trypsinogens is modified significantly in complexes with bovine pancreatic trypsin inhibitor (Kunitz). In both complexes, the chemical shift of the peak is similar to that of histidine-57 in spectra of the porcine trypsin-bovine pancreatic trypsin inhibitor complex [Markley, J. L., & Porubcan, M. A. (1976) *J. Mol. Biol. 102,* 487-509]. Calculations based on the environment of histidine-57 in the X-ray crystallographic structure of bovine trypsinogen [Fehlhammer, H., Bode, W., & Huber, R. (1977) *J. Mol. Biol. 111,* 415-438] show that the unusual NMR deshielding of the histidine-57 $C^{\epsilon 1}$ protons in trypsinogen results from magnetic anisotropy about the nearby carbonyl group of serine-214. The ^1H NMR data indicate that the environment and pK' of histidine-57 are different in trypsinogen and trypsin.

Trypsin, an important and extensively studied enzyme, is synthesized and stored by the pancreas in an inactive form known as trypsinogen. This precursor or zymogen is released into the duodenum and is activated by the specific action of another proteinase, enterokinase. Trypsin then acts to catalyze the conversion of additional trypsinogen to trypsin as well as initiating a cascade of other zymogen activations which are necessary for the production and maintenance of proper enzyme levels in the digestive tract.

Trypsinogen and trypsin have been the subject of numerous investigations throughout the years. Early chemical modification studies implicated the involvement of a histidyl and a seryl residue in the catalytic mechanism of trypsin and other

† From the Biochemistry Division, Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received April 24, 1978.* A preliminary account of this study has been presented (Porubcan et al., 1977). Supported by the National Institutes of Health Grants GM 19907 to the Purdue Research Foundation, and RR 00292 to the NMR Facility for Biomedical Studies, Carnegie-Mellon University where the 250-MHz ^1H NMR spectroscopy was carried out.
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