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High Resolution Nuclear Magnetic Resonance Studies of the Active Site of Chymotrypsin

II. Polarization of Histidine 57 by Substrate Analogues and Competitive Inhibitors

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The proton nuclear magnetic resonance signal of the His57–Asp102 hydrogen bonded proton in the charge relay system of chymotrypsinogen A and chymotrypsin A_δ has been monitored to determine the influence of substrate analogues and competitive inhibitors on the electronic state of the active site regions. Borate ion, benzene boronic acid and 2-phenylethylboronic acid, when bound to chymotrypsin at pH 9·5 shift the resonance position of the His–Asp hydrogen bonded proton to −15·9, −16·3 and −17·2 parts per million, respectively. These positions are intermediate between the low pH position in the free enzyme of −18·0 parts per million and the high pH position of −14·9 parts per million. The presence of these analogues prevents the His–Asp proton resonance from titrating in the region of pH 6 to 9·5. Similar low field shifts are observed for the hydrogen bonded proton resonance of subtilisin BPN′ when complexed with these boronic acids. The results support the chemical and crystallographic data which show that negatively charged tetrahedral adducts of the boronic acid substrate analogues are formed at the active sites of these enzymes. When combined with similar nuclear magnetic resonance data for the binding of *N*-acetyl-L-tryptophan to chymotrypsin A_δ, they suggest that a direct interaction occurs between the active site histidine and the atom occupying the leaving group position of the substrate, presumably a hydrogen bond.

The His–Asp proton resonance was also monitored in complexes of chymotrypsin A_δ with bovine pancreatic trypsin inhibitor over the pH range 4 to 9. In the complex the low field proton resonance had a field position of −14·9 parts per million over the pH range 4 to 9 indicating that His57 is in the neutral form, similar to the active enzyme at high pH.

1. Introduction

Nuclear magnetic resonance studies of the hydrogen bonded protons at the active site of chymotrypsin A_δ and in the same region of chymotrypsinogen A have recently been reported (Robillard & Shulman, 1972,1974) as part of an effort to determine the impact of the "charge relay" system on the overall catalytic activity of the serine esterase (Blow *et al.*, 1969). Using high resolution n.m.r.† at 220 MHz a resonance from an exchangeable proton was observed at extremely low fields for H_2O solutions of chymotrypsinogen A, chymotrypsin A_δ, trypsinogen, trypsin, subtilisin BPN and α-lytic protease. Detailed n.m.r. studies of this resonance in chymotrypsinogen A and chymotrypsin A_δ, including covalent chemical modifications of the protein,

† Abbreviations used: n.m.r., nuclear magnetic resonance; PTI, pancreatic trypsin inhibitor.

has enabled us to assign this resonance to the proton on the N(1) nitrogen of His57 which is in a strong hydrogen bond with the carboxylate of Asp102. In the same study we demonstrated that the N(3) nitrogen of His57 is also hydrogen bonded to the hydroxyl group of the active site Ser195, thereby supporting the existence of the charge relay system proposed by Blow *et al.* (1969).

In the present paper we report the response of the same low field proton resonance to competitive inhibitors and substrate analogues bound at the active site of chymotrypsin. It is well known that boronic acid substrate analogues and borate ions bind reversibly to chymotrypsin (Antonov *et al.*, 1968,1970; Philipp & Bender, 1971; Koehler & Lienhard, 1971). Their strong association constants were the first indication that they were bound at the active site as tetrahedral transition state analogues. This proposal has recently been supported by X-ray crystallographic studies on subtilisin–boronic acid complexes (Birktoft & Kraut, personal communication). Furthermore, the X-ray studies show that one of the boron oxygens is close enough to form a hydrogen bond with the N(3) nitrogen of His57. Similar X-ray crystallographic observations have been made for the complexes of chymotrypsin with *N*-formyl-L-tryptophan and *N*-formyl-L-phenylalanine in which one of the carboxylate oxygens is within hydrogen bond distance of the same nitrogen of His57 (Steitz *et al.*, 1969). Presumably this atom occupies a position analogous to the amide leaving group of a conventional peptide substrate.

Interactions between charged groups on the substrate and the imidazole ring of His57 should perturb the electron distribution of this ring which should shift the proton resonance at the N(1) position. Such perturbations have been observed and are reported below. They are interpreted in terms of the function of the charge relay system during the catalytic pathway.

2. Materials and Methods

(a) *Materials*

Enzyme. Bovine chymotrypsinogen A and bovine chymotrypsin A_δ (code CGC and CDD, respectively) were purchased from Worthington Biochemicals; bovine pancreatic trypsin inhibitor (type 1-P) was purchased from Sigma Chemicals and used without further purification. Subtilisin BPN′ (Nagarse) was purchased from Enzyme Development Corporation, 2 Penn Plaza, New York, N.Y. It was dissolved in 0·01 M-$CaCl_2$, pH 6, and chromatographed on a column of Sephadex G75 in 0·01 M-$CaCl_2$, pH 6, at 5°C and lyophilized immediately. 2-Phenylethyl boronic acid was generously supplied by G. Lienhard, Dartmouth University. All other chemicals were reagent grade and used without further purification.

(b) pH *measurements*

All measurements were made at room temperature using a Radiometer pH M-26 equipped with a combined glass electrode.

(c) *Nuclear magnetic resonance spectra*

Proton n.m.r. spectra were taken at 220 MHz with a Varian Associates HR220 n.m.r. spectrometer. The magnetic fields for the resonances are reported in terms of parts per million (p.p.m.) downfield from the fields required for the resonance of DSS (2,2-dimethyl-2-silapentane-5-sulfonate). Temperature was maintained constant to ± 1 deg. C with the Varian variable temperature accessory. The n.m.r. radio frequency power was 40 db (ethyl benzene quartet begins to saturate between 14 and 15 db) and the audio frequency

response was 4 Hz. To improve signal-to-noise all spectra were an average of 250 to 500 scans at 50 ms per channel using a Fabri-Tek 1024 computer. The pH of each sample was measured before and after each run. Only those data in which the pH drift was less than $\pm 0{\cdot}05$ pH unit were used.

3. Results

(a) *Influence of substrate analogues and non-competitive inhibitors on the His–Asp proton resonance*

Chymotrypsin–boronic acid complexes

Figure 1 shows the low field resonance in chymotrypsinogen A at pH 9·5 as a function of the borate salt concentration. It is clear that the resonance is shifted to lower field in the presence of borate. Identical behavior for this resonance in

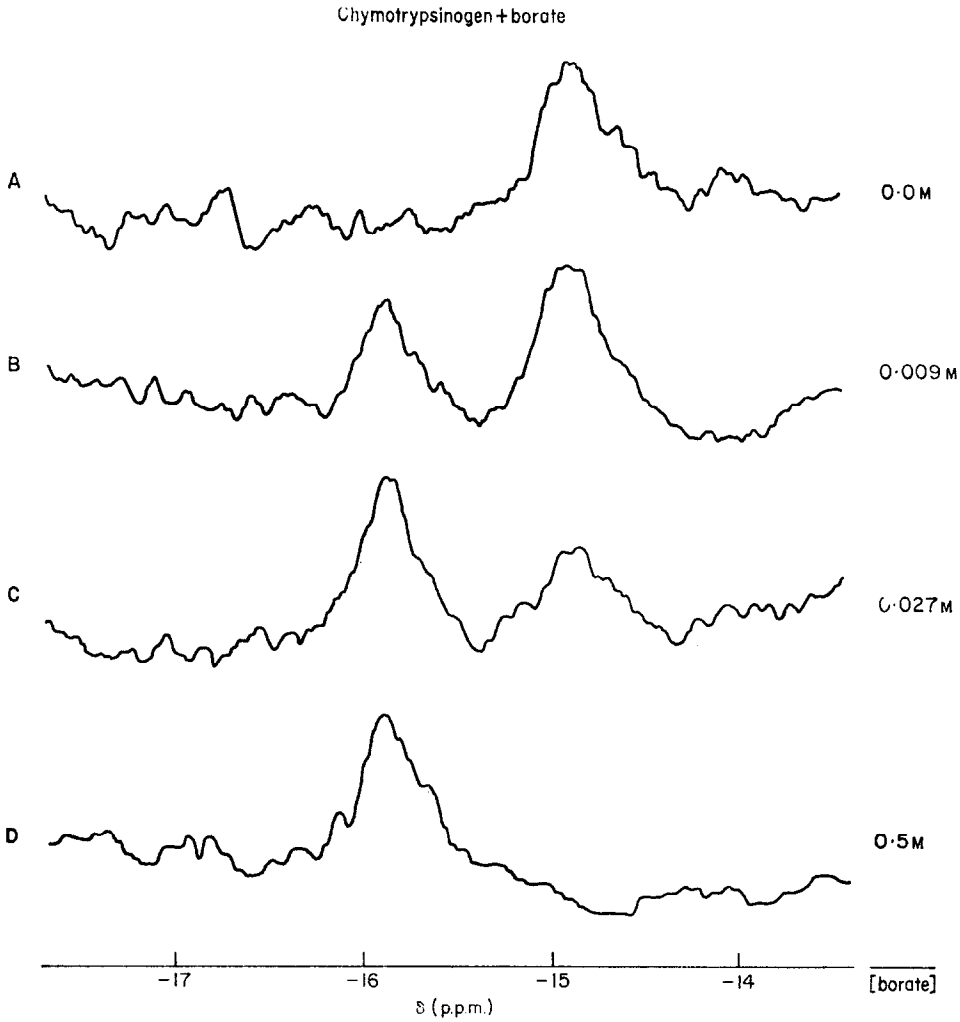


FIG. 1. 220 MHz proton n.m.r. spectra of chymotrypsinogen A and various concentrations of sodium borate. The experiments were carried out at 4°C, pH 9·5, in 0·5 M-sodium carbonate $[E] = 3{\cdot}5 \times 10^{-3}$ M/l.

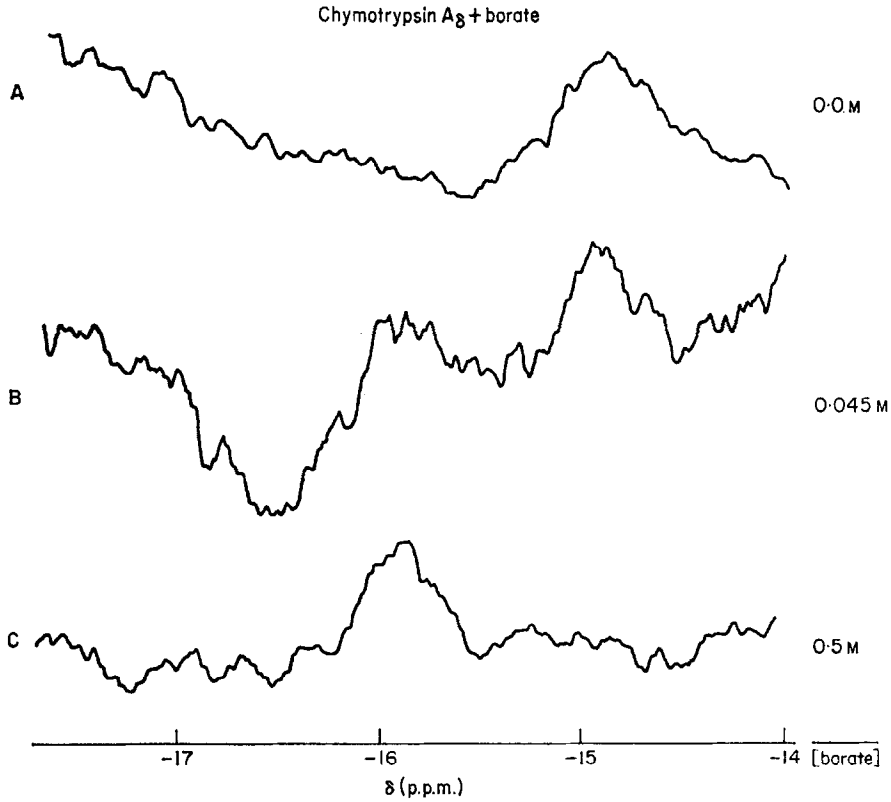


FIG. 2. 220 MHz proton n.m.r. spectra of chymotrypsin A_δ and various concentrations of sodium borate. [E] = $2{\cdot}9 \times 10^{-3}$ M/l. The temperature, pH and carbonate concentrations are as stated in Fig. 1.

chymotrypsin A_δ is seen in Figure 2. In the absence of borate the resonances are located at −15·0 p.p.m. and −14·9 p.p.m. for chymotrypsinogen A and chymotrypsin A_δ, respectively. At high borate concentrations these resonances disappear and new peaks appear at −16·0 and −15·9 p.p.m. At intermediate borate concentrations, both peaks are present. The observed effect is specific to borate ion and not to ionic strength because no shifts were observed when Tris or carbonate salts were used in place of borate. Furthermore, neither Tris or carbonate prevented the borate induced shift when chymotrypsin–borate complexes are measured in the presence of these salts.

Since borate ions presumably bind to many locations on the enzyme molecule, it is possible that the observed shift results from binding somewhere other than at the active site region. This possibility, however, has been eliminated by a study of the effect of borate on the field position of the His–Asp proton resonance in the alkylated derivatives Tos-PheCH_2Cl-chymotrypsin A_δ and *z*-Gly, Leu, Phe-chymotrypsin A_δ (Robillard & Shulman, 1974). The resonance field position is unaffected by borate

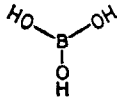
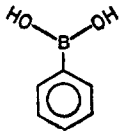
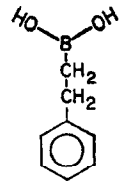
ions up to 0·5 M at pH 9·5 in these derivatives, while in the native enzyme molecule 0·1 M-borate ion is sufficient to shift the same resonance to lower fields.

When borate ions are replaced by the more specific boronic acid substrate analogues, benzene boronic acid and 2-phenylethyl boronic acid, even more extensive shifts of the resonance are observed in chymotrypsin A_δ. The results presented in Table 1 show that, at pH 9·5 in carbonate buffer, benzene boronic acid shifts the His–Asp proton resonance 1·55 p.p.m. to lower fields while 2-phenylethyl boronic acid causes a shift of 2·3 p.p.m. in the same direction. Although complexes of borate ion with chymotrypsin A_δ and chymotrypsinogen A result in identical shifts, 1·0 p.p.m. in each case, the more specific substrate analogues induce much smaller shifts in chymotrypsinogen than in the active enzyme.

In both native chymotrypsinogen A and chymotrypsin A_δ the His–Asp proton resonance shifts approximately 3·0 p.p.m. upon changing pH with the midpoint of this titration at pH 7·5 (Robillard & Shulman, 1972,1974). However, in the presence of the boronic acid substrate analogues the field position of this resonance is insensitive to pH. The shift induced upon binding the boronic acids at pH 9·5 remains

TABLE 1

Line shifts of the His–Asp hydrogen bonded proton resonance in chymotrypsinogen A and chymotrypsin A_δ in the presence of saturating amounts of inhibitor

δ (p.p.m., DSS) −18	−17	−16	−15	−14	Enzyme	Inhibitor	K_i (M) pH 8
			15·0		Chtgen A	None	—
			14·9		Cht A_δ	None	—
		16·0			Chtgen A		—
		15·9			Cht A_δ		1×10^{-1} (a)
	16·1				Chtgen A		—
	16·3				Cht A_δ		$1{\cdot}9\times10^{-4}$ (b)
		15·7			Chtgen A		—
	17·2				Cht A_δ		4×10^{-5} (c)

K_i for the inhibitors are taken from the literature: (a) Antonov *et al.* (1968); (b) Philipp & Bender (1971); (c) Koehler & Lienhard (1971).

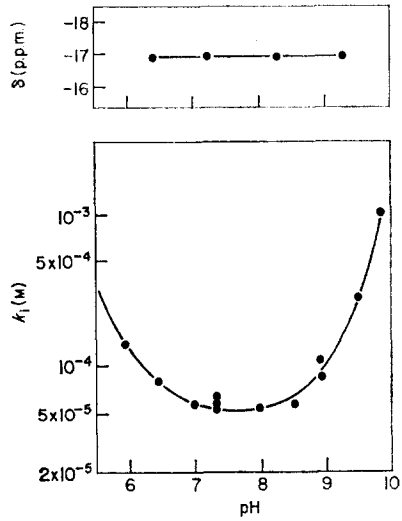


FIG. 3. Comparison of the pH dependence of K_i for 2-phenylethyl boronic acid inhibition of chymotrypsin (Koehler & Lienhard, 1971) with the pH dependence of the n.m.r. resonance field position for the complex of 2-phenylethyl boronic acid and chymotrypsin. The inhibitor concentration in the n.m.r. experiments was 0·075 M/l and the temperature was maintained at 3°C.

constant throughout the same pH range in which the inhibitor is strongly bound to the enzyme. The lower curve in Figure 3 presents the pH dependence of the binding of 2-phenylethyl boronic acid to chymotrypsin A_δ (Koehler & Lienhard, 1971). The upper half of Figure 3 is a plot of the pH dependence of the n.m.r. shift for the His–Asp proton resonance in the same boronic acid complex. Under the experimental conditions the inhibitor concentration, 0·075 M, was sufficient to saturate the enzyme binding site in the pH region 6 to 9·5. As Figure 3 shows, the n.m.r. shift is independent of pH under these conditions.

(b) *Subtilisin BPN′–boronic acid complexes*

As will be evident in the Discussion, the interpretation of the boronic acid-induced shifts in chymotrypsin A_δ is based, in large part, upon the X-ray, crystallographic studies of subtilisin BPN′–boronic acid complexes (Kraut & Birktoft, personal communication). The use of subtilisin X-ray data to interpret chymotrypsin n.m.r. data might appear to be overworking the concept of homology between the active sites of these two enzymes. Therefore experiments similar to those just reported were carried out with subtilisin BPN′. The low field proton n.m.r. resonance in subtilisin was monitored in the presence of benzene boronic acid and 2-phenylethyl boronic acid. In Figure 4 the n.m.r. spectra of these complexes are presented and compared with the n.m.r. spectra of chymotrypsin complexed with the same substrate analogues. Benzene boronic acid and 2-phenylethyl boronic acid induce identical chemical shifts for the low field proton resonance in subtilisin. Furthermore the chemical shifts in subtilisin are identical to those observed in complexes of chymotrypsin A_δ with 2-phenylethyl boronic acid. Figure 5 illustrates the pH dependence of the field position of this resonance in the subtilisin–boronic acid complexes. It is independent of pH over the range 6 to 8·5 in which the boronic acids are tightly

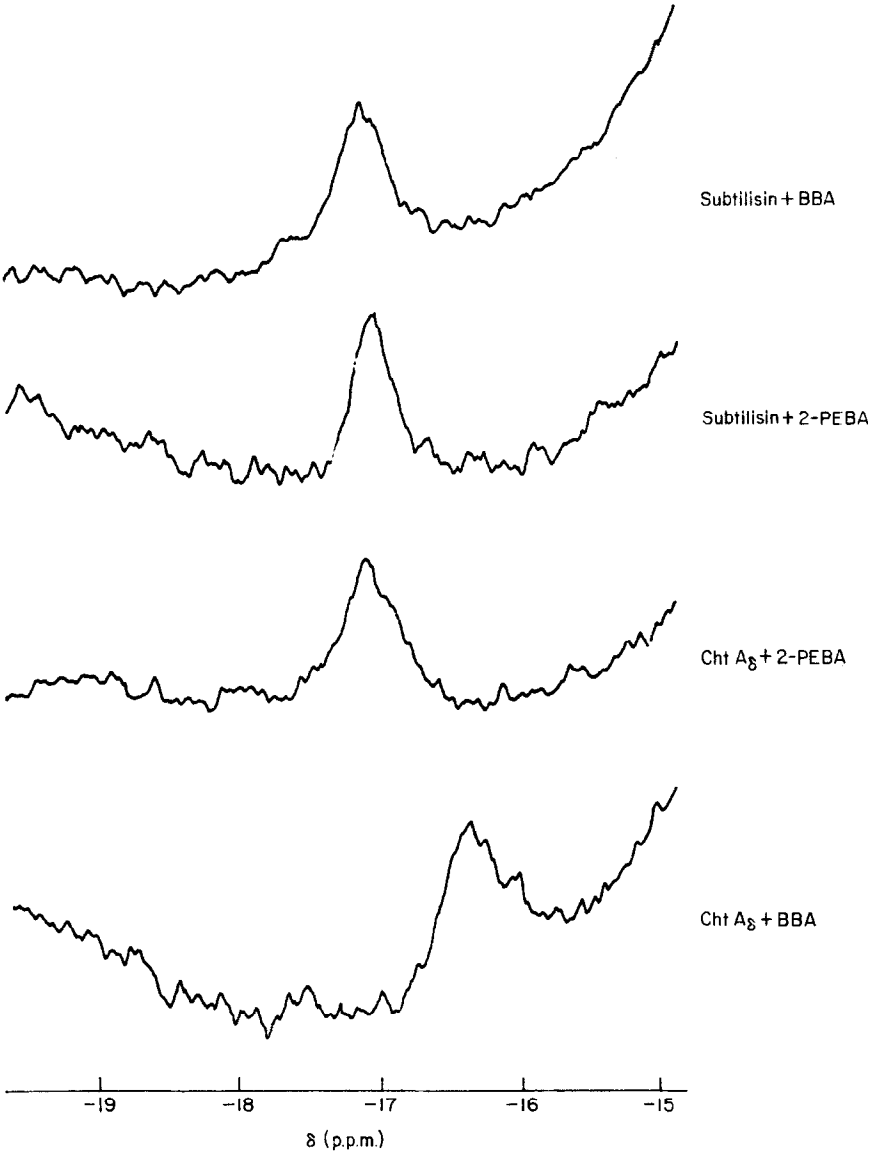


FIG. 4. 220 MHz proton n.m.r. spectra of chymotrypsin A_δ and subtilisin BPN′ complexes with 2-phenylethyl boronic acid (PEBA) or benzene boronic acid (BBA) Cht, chymotrypsin. Enzyme concentrations: 3 to 4 mM-benzene boronic acid concentration: 0·2 M-2-phenylethyl boronic acid concentration: 0·075 M. Temperature +3°C, pH 8 to 9.

bound to the enzyme. When compared with the chymotrypsin data in the same Figure, the same behavior for the inhibitor complexes with both enzymes is evident. The similarities in the response of the His–Asp proton in chymotrypsin and subtilisin to the boronic acid substrate analogues, appear to justify using subtilisin X-ray data as a guide to interpreting the chymotrypsin n.m.r. data in this particular case.

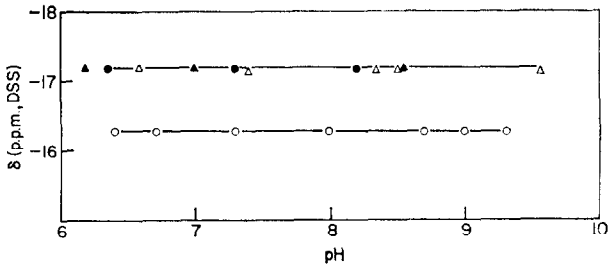


FIG. 5. n.m.r. resonance position as a function of pH, (○) Chymotrypsin and benzene boronic acid; (△) chymotrypsin and 2-phenylethyl boronic acid; (●) subtilisin and benzene boronic acid; (▲) subtilisin and 2-phenylethyl boronic acid. Enzyme and inhibitor concentrations were as given for Fig. 4.

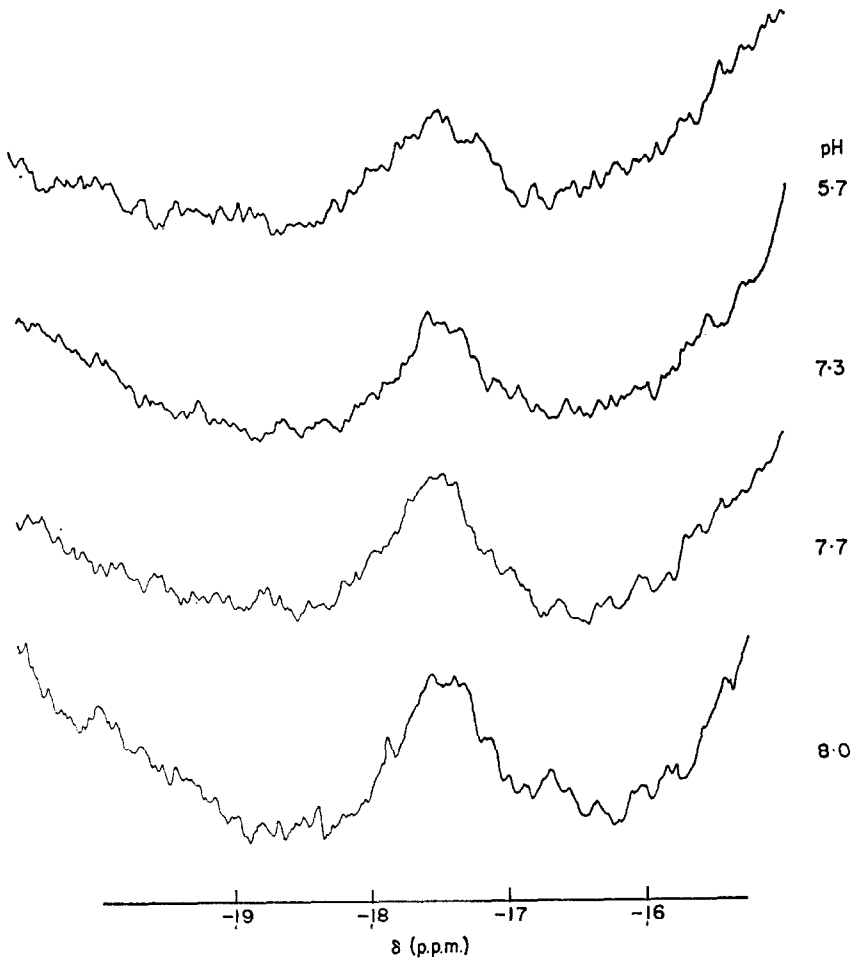


FIG. 6. 220 MHz n.m.r. spectra of chymotrypsin and *N*-acetyl-L-tryptophan. Enzyme concentration was 3 to 4 mM. Inhibitor concentration was 0·1 M below pH 7·5 and 0·5 M above pH 7·5. Temperature was 3°C.

(c) *Chymotrypsin–carboxylic acid complexes*

The borate and boronate induced shifts of the His–Asp proton resonance seem to show that the active site region of the enzyme can be quite sensitive to the presence of charged inhibitors. This observation has been tested further by monitoring the effect of carboxylic acid inhibitors on the position of the His–Asp proton resonance. Johnson & Knowles (1966) have demonstrated, by equilibrium dialysis techniques, that *N*-acetyl-L-tryptophan bound more strongly to the low pH form of chymotrypsin

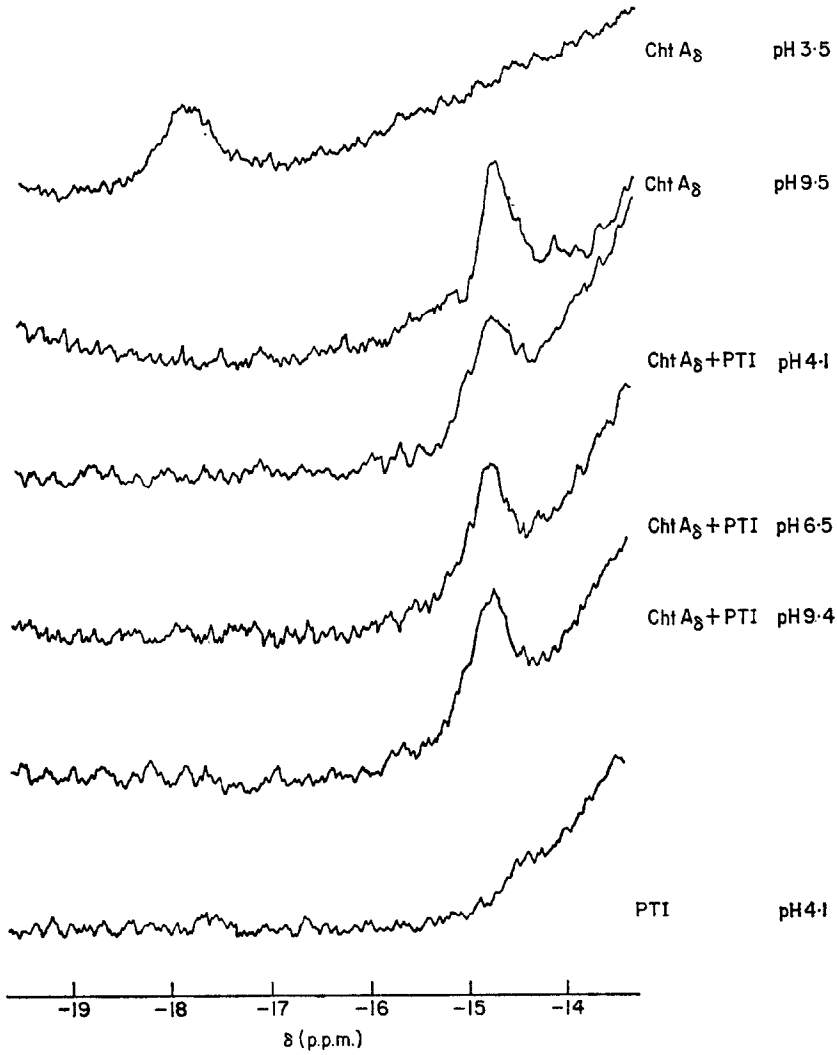


FIG. 7. 220 MHz n.m.r. spectra of chymotrypsin A_δ (Cht), bovine pancreatic trypsin inhibitor and chymotrypsin A_δ–PTI complexes. Temperature was 3°C. Chymotrypsin concentration was 3 to 4 mM. Pancreatic trypsin inhibitor concentration was 3 to 4 mM. Chymotrypsin–PTI complex concentration was ≈3 mM. The complex was prepared by dissolving 10 mg PTI in 0·25 ml of water (pH 4) followed by 25 mg chymotrypsin A_δ.

A_a than to the high pH form. A k_1 of ~2 mM was measured between pH 2·2 and 7 while a value of ~14 to 24 mM was observed between pH 8 and 8·3. The binding therefore was dependent on a group ionizing with a pK_a of 7·3. We have measured the n.m.r. field position of the His–Asp proton resonance in chymotrypsin under conditions where the active site was saturated with *N*-acetyl-L-tryptophan (i.e. 0·1 M in *N*-acetyl-L-tryptophan from pH 5·5 to 7·5 and 0·5 M from pH 7·5 to 8·5). The spectra are presented in Figure 6. Under these conditions the resonance field position is −17·5 p.p.m. and it remains constant across the pH range 5·4 to 8·5. This pH independence is similar to that observed in the chymotrypsin and subtilisin boronate complexes.

(d) *Chymotrypsin–trypsin inhibitor complex*

The X-ray crystal structure and the chemical studies on chymotrypsin and subtilisin boronic acid complexes have demonstrated that the boronic acids are present at the active site as stable tetrahedral adducts of boronate anions (Kraut & Birktoft, personal communication; Koehler & Lienhard, 1971). When bovine pancreatic trypsin inhibitor (PTI) is complexed with chymotrypsin the X-ray crystallographic data suggest that a similar stable tetrahedral adduct is formed (Rühlmann *et al.*, 1973). In this case the tetrahedral geometry occurs about the carbonyl carbon of Lys15I which is in a covalent bond with Ser195 of chymotrypsin.† Because of the rather striking response of the His–Asp proton resonance to the presence of the bound boronic acids, the resonance was also monitored in complexes of chymotrypsin plus PTI. The spectra of these complexes at three different pH values are compared, in Figure 7, with spectra of free chymotrypsin A_δ and free PTI. The n.m.r. spectra of the complexes show a resonance in the low field region at −14·9 p.p.m. which again is invariant with pH. The resonance is not present in free PTI but it is present at an identical field position in free chymotrypsin A_δ at high pH.

4. Discussion

It has been demonstrated by kinetic methods that the chymotrypsin-catalyzed hydrolysis of amides and peptides proceeds *via* a tetrahedral intermediate species of the substrate just as in the hydrolysis of amides in solution (Jencks, 1969; Fersht, 1971; Fersht & Requena, 1971; Caplow, 1969; Lucas *et al.*, 1973). The kinetic data for the existence of this intermediate has been supported by X-ray crystallographic investigations showing structural features at the active sites of chymotrypsin and subtilisin BPN′ which could stabilize such an intermediate (Henderson *et al.*, 1971; Henderson, 1970; Robertus *et al.*, 1972). Whether such an intermediate is stable enough to accumulate is still in contention. Nevertheless, it is obvious that the placement of such a charged species at the active site, with its rather rigid stereochemical requirements, may have a profound effect on the state of protonation of the amino acid side chains directly involved in the catalytic process. One way to study this intermediate catalytic state in detail is to use stable tetrahedral transition state analogues. Organoboron compounds as well as borate itself can exist in either of two general configurations. In one the boron atom is trigonal coplanar with sp^2 hybridization, having empty 2p orbitals above and below the BO_3 plane. Thus,

† Amino acid residues of pancreatic trypsin inhibitor are designated by I.

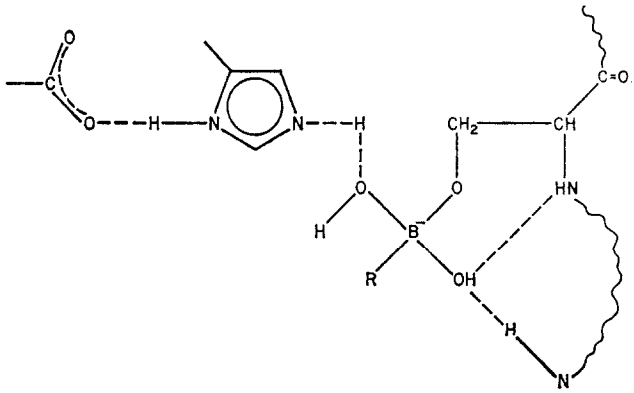


FIG. 8. Schematic representation of the active site region of chymotrypsin complexed with boronate inhibitors. The information presented here is a combination of n.m.r. data and X-ray crystallographic data from subtilisin–boronate complexes provided by Kraut & Birktoft (personal communication).

being electron deficient the boron is capable of adding a fourth ligand and converting to the second configuration which is tetrahedral with sp^3 hybridization (Gerrard, 1961; Edwards *et al.*, 1955). Because of this ability to form stable tetrahedral adducts Koehler & Lienhard (1971) examined the boronic acids as possible analogues of the tetrahedral transition state and suggested that their kinetic data on 2-phenylethyl boronic acid could be explained by the formation of a tetrahedral adduct. Complexes of 2-phenylethyl boronic acid and benzene boronic acid with subtilisin BPN′ have been shown by recent X-ray crystallographic studies to exist as the tetrahedral adduct (Kraut & Birktoft, personal communication). The boron is covalently attached to the active site serine while one of its oxygens is hydrogen bonded to backbone amide groups in the "oxy-anion hole" and the other oxygen is hydrogen bonded to N(3) nitrogen of the active site histidine as shown schematically in Figure 8. We propose that the n.m.r. studies of the boron complexes in both chymotrypsin and subtilisin can be interpreted in terms of this structure and that the n.m.r. shifts can be used to understand charge distribution within the complex. It should be noted that the X-ray studies were done on the boronic acid inhibitors and not on borate itself. It is possible that borate could bind covalently to the histidine. However because of the arguments presented in Section (b)(3) of the Discussion we feel that the X-ray data on the boronic acid inhibitors describe the structure of the borate–enzyme complex as well.

(a) *Origin of the inhibitor induced nuclear magnetic resonance shifts*

The discrete shift in the His–Asp proton resonance with borate suggests that the borate reaction with the enzyme is slow on the n.m.r. time scale. This means that the lifetime of the borate–chymotrypsin A_δ and borate–chymotrypsinogen A complexes are long compared to the linewidths, i.e. $\tau \gg (\pi\delta\nu)^{-1}$ or $\tau \gg 3\times10^{-3}$ seconds. If the reaction is represented as a simple equilibrium

$$\mathrm{E} + \mathrm{B} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{EB}$$

then the equilibrium constant is

$$K = \frac{[E][B]}{[EB]} = \frac{k_{21}}{k_{12}}.$$

Since intermediate borate concentrations show no additional broadening of the enzyme and zymogen NH resonances (Figs 1 and 2) we conclude that the forward rate of borate is not fast enough to broaden the lines. Hence

$$\frac{1}{[E]_f}\frac{\partial[E]}{\partial t} = k_{12}[B]_f = \frac{1}{\tau} \ll \pi\delta\nu$$

or

$$k_{12} \ll 3\times10^4 \text{ M}^{-1}\text{ l s}^{-1}$$

for a free borate concentration of $[B]_f \approx 0{\cdot}01$ M, and $\delta\nu \sim 10^2$ Hz. The rate of formation of simple enzyme–inhibitor complexes occurs orders of magnitude faster (k_{12} for the reaction of proflavin with chymotrypsin A_α is $\approx 10^8$ M^{-1} l s^{-1} (Havsteen, 1967)). Therefore this rather slow rate of borate binding is consistent with the formation of a covalently bound complex to the enzyme. The total concentrations of enzyme and borate are known and [E] and [EB] can be determined from the areas under the two resonance peaks. Hence the apparent equilibrium constants for the borate complexes can be determined. At pH 9·5 in 0·5 M-sodium carbonate we calculate that k equals $1{\cdot}3\times10^{-2}$ M/l and $5{\cdot}6\times10^{-2}$ M/l for the borate–chymotrypsinogen and borate–chymotrypsin A_δ complexes, respectively. The value for chymotrypsin A_δ is within a factor of two of the inhibition constant, k_1, for chymotrypsin A_α, 1×10^{-1} M/l, determined by competitive inhibition studies (Antonov *et al.*, 1970). The binding constant to chymotrypsinogen has not been reported.

Three lines of evidence in the above experiments demonstrate that the shifts induced by borate and the boronic acids result from a direct interaction with the active site region.

(1) The downfield shifts observed with the active enzyme–inhibitor complexes increase monotonically with the inhibition constants determined by competitive inhibition measurements (see Table 1).

(2) The equilibrium constant determined from the n.m.r. measurements of the borate–chymotrypsin A_δ complex is within a factor of two of the inhibition constant in chymotrypsin A_α measured by competitive inhibition.

(3) In derivatives of chymotrypsin A_δ which have been alkylated at the N(3) position of His57, the His–Asp proton resonance is not shifted by borate. This indicates that borate competes for the region occupied by part of the alkylating group.

(b) *The variation in the nuclear magnetic resonance shifts*

Having established that the shifts of the His–Asp proton resonance arise *via* a direct interaction of the boronates with the active site region, it is now necessary to consider the possible mechanisms which would generate a different chemical shift for each inhibitor studied. There are three such mechanisms.

(1) A conformational change could result upon binding boronates which would perturb the active site residues so as to cause the observed shifts. Such an explanation is unlikely since it would necessitate five different conformational changes, or, at

least, five different degrees of a single conformational change, in chymotrypsin and chymotrypsinogen.

(2) The different chemical shift for each enzyme–inhibitor complex could be a function of the electronic charge on the inhibitor. This alternative is likewise unacceptable since the pK_a values for the ionization of the inhibitors, which are a measure of their charge, vary in a different order from the inhibitor-induced change in chemical shift (Yabroff *et al.*, 1934). Furthermore, if charge distribution was the principal mechanism for the variation in the chemical shifts one would expect to observe the same shift for each inhibitor whether bound to chymotrypsin A_δ or to chymotrypsinogen A and this is not the case.

(3) The most probable mechanism of these shifts includes both the rigid stereochemical requirements of the active site region and the electronic structure of the tetrahedral complex. When an aromatic binding site is not required to bind the inhibitor, as in the case of borate, the inhibitor-induced shift is the same for chymotrypsinogen and chymotrypsin A_δ complexes. In this case the borate ion is presumably free to take the optimum position for the bond and the observed shift depends only upon the electronic nature of borate. However, when the inhibitor possesses an aromatic moiety and becomes more like a substrate, as witnessed by the decrease in k_1, progressively larger shifts occur in the active enzyme, but smaller shifts occur in chymotrypsinogen which lacks most of the substrate binding site (Freer *et al.*, 1970). Thus, the difference in orientation of the inhibitor with respect to the active site residues is responsible for the difference in the chemical shifts observed in these complexes.

This combined effect of structure and electronic properties upon the resonance is observed in the n.m.r. data of the subtilisin BPN′–boronic acid complexes (Figs 4 and 5). The His–Asp proton resonance in subtilisin is shifted to the identical position in both the 2-phenylethyl boronic acid and benzene boronic acid complexes. In keeping with this observation, the X-ray crystallographic studies of the subtilisin–boronic acid complexes show that these two boronic acids occupy identical positions at the active site (Kraut & Birktoft, personal communication). Therefore, when the orientations of the inhibitors are the same, the n.m.r. resonance positions in the complexes are the same. This leads us to predict that in chymotrypsin A_δ these two boronic acid complexes will have slightly different structures.

(c) *Interpretation of the nuclear magnetic resonance shifts*

Johnson & Knowles (1966) have demonstrated that neutral inhibitors such as *N*-acetyl-D-tryptophan amide bind to chymotrypsin independently of pH over the range pH 2·2 to 9·6. On the other hand the binding of negatively charged inhibitors, such as *N*-acetyl-D- or L-tryptophan, depended on the ionization of a group in the free enzyme with a pK_a of $\sim$7·3. Therefore they suggested that the active site of the free enzyme carried a net charge of -1 at pH $> 7{\cdot}5$. This interpretation is consistent with the n.m.r. data and with the structure shown in Figure 9. Fersht & Sperling (1973) have shown that a residue with a $pK < 3$, becomes exposed and protonates upon denaturation at pH > 3. This too is consistent with Figure 9 since, upon denaturation, the individual residues become available for protonation which

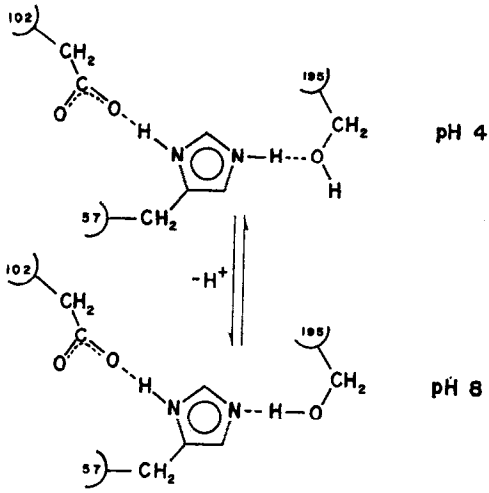


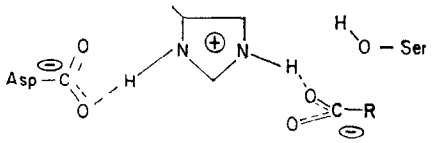
FIG. 9. Schematic representation of the active site region of chymotrypsin at low and high pH.

for $3 < pH < 6$ should result in one additional proton binding site. Turning to the boronate binding Koehler & Lienhard (1971) observed the same type of pH dependence for the binding of 2-phenylethyl boronic acid to chymotrypsin as Johnson & Knowles (1966) observed, but with a slightly lower pK. They proposed that a net charge of -1 was carried in the active site region of the boronate–enzyme complex. Their preferred structure for this complex, which is supported by the recent X-ray crystal structure (Kraut & Birktoft, personal communication) is shown in Figure 8. These observations require that the boronic acid raises the pK of the His–Asp region so that in the boronate complex there is a proton at the N(3) of His57, which is presumably held there by a hydrogen bond. This is consistent with the observations that the positions for the His–Asp proton resonance in the chymotrypsin–boronate complex are independent of pH as shown in Figures 3 and 5 and by the positions of the resonance (see Table 1) which are intermediate between those observed for the high and low pH forms of the enzyme (cf. accompanying paper). These intermediate positions presumably reflect the charge perturbation on the imidazole ring arising from different interactions of the boronate oxygens with the N(3) of His57 through the hydrogen bond (Fig. 8). It is our assumption that the field position of the His–Asp proton resonance reflects this charge perturbation based upon the results on the unmodified enzyme where the resonance shifts from $-15{\cdot}0$ to $-18{\cdot}0$ p.p.m. as the pH is lowered.

With these assumptions we can interpret the present data so as to give qualitative information about the position of the proton in the N(3) His–borate hydrogen bond. In the borate complexes the resonance position of $-16{\cdot}0$ p.p.m. suggests that the N(3) proton is fairly close to the borate oxygen, possibly reflecting the greater attraction of the borate for a proton. In the strongly bound 2-phenylethyl boronic acid the resonance position of $-17{\cdot}2$ p.p.m. indicates that the proton has moved closer to His57, presumably because the bond is weakened by the constraints placed upon the inhibitor's position by the aromatic binding pocket.

While borate and boronate anions form covalent complexes at the active site and undergo a change in the hybridization state of the boron, carboxylate anions do not.

It is known from the X-ray crystallographic studies that *N*-formyl-L-tryptophan and *N*-formyl-L-phenylalanine form non-covalent complexes with the active site of the enzyme and maintain a trigonal configuration about the carboxylate carbon. Nevertheless, in these complexes also one of the carboxylate oxygens is hydrogen bonded to the N(3) nitrogen of His57 (Steitz *et al.*, 1969). This is consistent with the data of Johnson & Knowles (1966) which demonstrates that the carboxylate anion of *N*-acetyl-L-tryptophan has a stronger affinity for the low pH form of the enzyme. Apparently a charge–charge interaction between imidazolium ion and the anionic inhibitor as shown in scheme 1 accounts for a substantial portion of the low pH affinity.

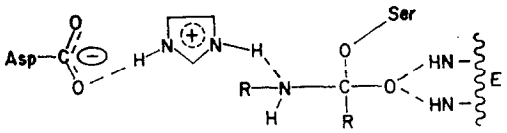


Scheme 1

Since this system is quite analogous to that presented in Figure 8 for the enzyme–boronic acid complexes, one would predict a similar response of the His–Asp proton resonance to the presence of *N*-acetyl-L-tryptophan. The field position of this resonance (−17·5 p.p.m.) and its invariance with pH as shown in Figure 6 confirm these predictions. From the calibration used for the boronic acid complexes, the resonance field position being close to −18·0 p.p.m. suggests that the histidine is close to the fully protonated state, but that it is not fully protonated, presumably because of a hydrogen bond to the carboxylate.

(d) *The transition state*

The bond-making and bond-breaking steps occurring in the transition state are accompanied by proton transfer from His57 to the leaving group of the substrate. In both the boronic acid and carboxylic acid chymotrypsin complexes we have demonstrated a direct interaction between the atom occupying a position analogous to the leaving group of a normal substrate and the proton at the N(3) position of His57. A similar structure for the normal peptide complex is presented in scheme 2.



Scheme 2

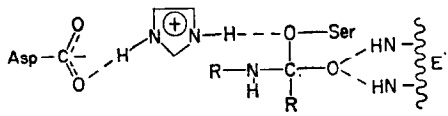
One important conclusion from the present experiments is that, in these tetrahedral complexes, His57 can be maintained at intermediate degrees of protonation as demonstrated by the intermediate field positions of the His–Asp proton resonance. This is in contrast to normal imidazole in solution which is either protonated or not, and shows no intermediate degrees of protonation. The ability of the histidine to assume intermediate degrees of protonation in these complexes suggests that in the tetrahedral state there may be an unusually shallow potential energy well for the

proton between the N(3) of His57 and the amide nitrogen of the substrate leaving group. It has been calculated (Merlet *et al.*, 1972) for the $[H_3N{-}H\ldots NH_3]^+$ system that when the distance between the heavy atoms is reduced, the potential energy barrier is decreased and eventually disappears. Furthermore the energy cost to eliminate this barrier and create just a single broad potential minimum is only 2·9 kcal. Therefore we suggest that upon formation of the tetrahedral intermediate the two hydrogen bonds formed between the substrate and the enzyme in the oxy-anion hole could provide the energy necessary to move the leaving group nitrogen close enough to the histidine so that the potential well of the N(3) proton between His57 and the leaving group nitrogen is made shallower, possibly going to a single minimum. Certainly the borate experiments demonstrate a dependence of the hydrogen bond potential upon binding of the aromatic moiety of the substrate. The variety of proton positions observed in the boronate complexes is more consistent with a shallow minimum than with the deep double minimum potential model of a normal hydrogen bond. This minimization of the potential energy barrier for proton transfer leads to a further understanding of the catalysis if the reaction proceeds via a concerted mechanism. In this case, first the substrate carbonyl approaches the serine and bond formation occurs with the carbonyl carbon assuming a tetrahedral geometry. Then we suggest that hydrogen bonds which form in the oxy-anion hole provide the energy required to push the leaving group nitrogen close enough to N(3) of His57. With the potential energy barrier for proton transfer to the leaving group minimized by this process, protonation of the leaving group nitrogen occurs in concert with peptide bond cleavage, and this rate limiting step is catalyzed by the accommodating nature of the hydrogen bonded proton.

These present experiments suggest that the function of the buried aspartate is to increase the polarizability of His57 so that together they serve as a charge reservoir, facilitating the uptake and release of protons during the catalytic process.

(e) *Chymotrypsin–pancreatic trypsin inhibitor complexes*

Finkenstadt & Laskowski (1965) have shown that chymotrypsin displays limited enzymatic activity towards the trypsin inhibitor molecule and proposed that the enzyme forms either a stable acyl intermediate or tetrahedral intermediate with the inhibitor. The recent X-ray data of Rühlmann *et al.* (1973) on the trypsin–PTI complex indicates that it is the tetrahedral species which is formed. Furthermore Rühlmann *et al.* (1973) also observed that the histidine is close enough to the serine oxygen to be hydrogen bonded, and used this observation to explain the stability of the tetrahedral adduct. In this view the hydrogen bond between the N(3) nitrogen of His57 and the serine oxygen stabilizes the tetrahedral adduct by preventing its proton from being transferred to the amide nitrogen of Ala16I as shown in scheme 3.



Scheme 3

However, after refinement of the X-ray crystal structure of the bovine trypsin–PTI complex Huber *et al.* (1973) reported a distance of 2·3 Å between the oxygen of Ser195

and the carbonyl carbon of Lys15I. This distance does not correspond to a true covalent bond distance which is ≈ 1·5 Å. While Huber *et al.* (1973) realize the possibility that the value of 2·3 Å could reflect a bimodal distance distribution between 1·5 Å and 3·0 Å with 2·3 Å being the mean value, they do not observe this distribution of electron density in their maps and therefore they prefer a single complex represented by a bond distance of 2·3 Å. The n.m.r. magnetic field position of the His–Asp proton resonance in the chymotrypsin–PTI complex differs from what would be predicted on the basis of scheme 3 where His57 is protonated and the resonance should occur between −17 and −18 p.p.m. as it does in the native enzyme at low pH. Instead in the complex the resonance occurs at −14·9 p.p.m. across the pH range 4 to 9·4 (Fig. 7) which is identical to that observed in the native enzyme at high pH where His57 is not protonated but hydrogen bonded to Ser195 (Robillard & Shulman, 1974). Thus, in light of the apparent uncertainty concerning whether or not the covalent bond is actually formed between Ser195 and the inhibitor, the n.m.r. data suggests that it is not. This interpretation of the n.m.r. results is further supported by recent findings of Lazdunski (personal communication) on the anhydrotrypsin–PTI complex. In those studies the active site serine of trypsin is converted to dehydroalanine thus preventing covalent bond formation with the inhibitor. Comparisons of the binding constant and the association and dissociation rate constants for the interaction of native or anhydrotrypsin with both native and reduced PTI show only very slight differences between native and modified trypsin suggesting that the active site serine plays very little, if any role in the stabilization of the enzyme inhibitor complex.

We would like to thank Dr G. Lienhard for supplying the 2-phenylethyl boronic acid as well as for his helpful discussions of the results. We are very grateful to Drs Kraut and Birktoft for making available their unpublished data without which the interpretations presented in this paper would not have been possible. We also appreciate the helpful comments of Dr W. P. Jencks, and an informative preprint from M. Lazdunski.

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