the binding sites. Since the monomer to monomer interactions in the polymer involve a large fraction of the total surface of the monomer, it seems unlikely that polymerization may occur without decreasing the number of the binding sites unless a very loose polymeric structure is formed as seems to be the case for actin polymerized in the presence of KCl. If this is true, polymerization induced by $MgCl_2$ must have led to a much more compact polymeric structure characterized by a further (20-fold) decrease of the binding constant for Tb^{3+}. This view is supported by the very recent finding of Crawford et al. (1980), who isolated, from guinea pig polymorphonuclear leukocytes, two different polymeric forms of actin which they called Mg-actin and K-actin. The structural differences of the two polymers could be the basis of functional differences. This possibility is now under study.

Acknowledgments

We are grateful to Ilan Gruenwald for the helpful support in performing some of the experiments. We thank Rinaldo Rescazzi of the Instituto Chimico of the Ferrara University for the atomic absorption determinations.

References

Barden, J. A., & Dos Remedios, C. G. (1979) *Biochem. Biophys. Res. Commun. 86*, 529–535.
Barden, J. A., & Dos Remedios, C. G. (1980) *Biochim. Biophys. Acta 624*, 163–173.
Bray, G. A. (1960) *Anal. Biochem. 1*, 279–285.
Brittain, H. G., Richardson, F. S., & Martin, R. B. (1976) *J. Am. Chem. Soc. 98*, 8255–8260.
Collins, J. H., & Elziga, M. (1975) *J. Biol. Chem. 250*, 5195–5920.
Crawford N., Hema, C., & Jackson, P. (1980) *Biochim. Biophys. Acta 626*, 218–233.
Dos Remedios, C. G., & Dickens, M. (1978) *Nature (London) 276*, 731–733.
Dos Remedios, C. G., Barden, J. A., & Valois, A. G. (1980) *Biochim. Biophys. Acta 624*, 174–186.
Gordon, D. J., Young, Y. Z., & Korn, E. D. (1976) *J. Biol. Chem. 251*, 7474–7479.
Grazi, E., & Magri, E. (1981) *FEBS Lett. 123*, 193–194.
Horrocks, W. D. W., Jr., & Sudnick, D. R. (1979) *J. Am. Chem. Soc. 101*, 334–340.
Kasai, M., Asakura, S., & Oosawas, F. (1962) *Biochim. Biophys. Acta 57*, 22–31.
Lo Scalzo, J., & Reed, G. H. (1976) *Biochemistry 15*, 5407–5413.
Lowry, O. H., Rosenbrough, N. J., Farr, A. A., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Oriol-Audit, C. (1978) *Eur. J. Biochem. 87*, 371–376.
Rich, S. A., & Estes, J. E. (1976) *J. Mol. Biol. 104*, 777–792.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Strzelzcka-Golaszewka, H., Prochniewicz, E., & Drabikowski, W. (1978) *Eur. J. Biochem. 88*, 219–227.

Proton Nuclear Magnetic Resonance Evidence for the Absence of a Stable Hydrogen Bond between the Active Site Aspartate and Histidine Residues of Native Subtilisins and for Its Presence in Thiolsubtilisins†

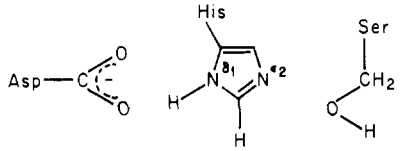
Frank Jordan* and László Polgár

ABSTRACT: The very low field proton nuclear magnetic resonance (^1H NMR) found in aqueous solutions of serine proteases and their zymogens is characteristic of the hydrogen bond between the imidazolium and aspartate groups of the catalytic triad: Ser-His-Asp [Robillard, G., & Shulman, R. G. (1972) *J. Mol. Biol. 71*, 507–511]. According to ^1H correlation NMR spectroscopic studies performed in 80/20 (v/v) $H_2O/^2H_2O$, no such resonance is found in native subtilisins (even at −2 °C and pH 6.0), but it is present in thiolsubtilisins and in the phenylboronic acid derivatives of the serine enzymes. The resonance was not visible in the mercuric or carboxamidomethyl derivatives of the thiol enzymes or in the phenylboronic acid–serine enzyme complex if the serine enzyme was first acylated with phenylmethanesulfonyl fluoride. The histidine at the catalytic site of thiolsubtilisin carries a positive charge between pH 5.6 and 8.4, in accord with previous data in favor of a mercaptide–imidazolium ion pair at the catalytic site. The charge distribution (− + −) at the active site of thiolsubtilisin and in the phenylboronic acid derivatives of the serine enzymes resembles that in the tetrahedral transition state formed between a serine enzyme and its substrate. Therefore, the stable hydrogen bond (found in the thiol enzyme and in the phenylboronic acid derivative of the serine enzyme) should be more important during catalysis than in the substrate-free enzyme.

X-ray diffraction studies on serine proteases, such as chymotrypsin, elastase, trypsin, and subtilisin, revealed the presence of an aspartate carboxyl group in the vicinity of the imidazole of the active site histidine residue [see Kraut (1977) for a review]. Blow et al. (1969) proposed that the triad of residues (aspartate, histidine, serine) at the catalytic center constitutes a "charge relay" that can conduct the electron pair from Asp to His to Ser, thereby enhancing the nucleophilicity of the catalytic serine oxygen. Such a relay of charge was questioned on chemical grounds (Polgár & Bender, 1969; Polgár, 1972; Rogers & Bruice, 1974). It was suggested by Polgár (1972) that the proton that is bound to the imidazole

† From the Department of Chemistry, Rutgers, The State University of New Jersey, Newark, New Jersey 07102 (F.J.), and the Institute for Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, H-1502, Hungary (L.P.). *Received May 4, 1981.* Work at Rutgers was supported by the Rutgers Research Council and the Charles and Johanna Busch Biomedical Grant. The Middle Atlantic NMR Facility at the University of Pennsylvania is supported by National Institutes of Health Grant RR-542.

0006-2960/81/0420-6366$01.25/0



nitrogen may be hydrogen bonded to but not transferred to the aspartate ion. It was further pointed out that such a hydrogen bond should be more important during the formation of the tetrahedral intermediate with the substrate, when the imidazole group is protonated, than in the substrate-free enzyme (Polgár & Bender, 1969). Proton nuclear magnetic resonance (^{1}H NMR) studies by Robillard & Shulman (1972, 1974a,b) also suggested only partial proton transfer through the hydrogen bond and emphasized the existence of this bond in the free enzyme. Hunkapiller et al. (1973) interpreted their carbon-13 nuclear magnetic resonance (^{13}C NMR) data obtained on specifically [^{13}C(2)]His-enriched α-lytic protease in favor of a complete proton transfer to aspartate ion at pH <7. By contrast, Bachovchin & Roberts (1978) examined the same enzyme specifically enriched in ^{15}N at the single histidine imidazole and demonstrated that the proton remains on the imidazole during the pH titration near pH 7. Porubcan et al. (1979) examined the pH dependence of the C(2)-H resonance of imidazole in α-lytic protease and reached a conclusion in accord with that of Bachovchin & Roberts (1978). Most recently, neutron diffraction studies were reported on isopropylphosphoryltrypsin (Kossiakoff & Spencer, 1980). It was concluded that in the catalytic cycle His rather than Asp is the protonated species.

In order to evaluate the importance of the hydrogen bond between the imidazole and the carboxylate at the catalytic center, we have undertaken studies comparing subtilisin and thiolsubtilisin. The latter can be prepared from subtilisin by chemical modification (Polgár & Bender, 1966; Neet & Koshland, 1966). Thiolsubtilisin contains a cysteine rather than a serine at the active center. The cysteine residue forms a mercaptide–imidazolium ion pair with the neighboring histidine residue (Polgár, 1974). Therefore the charge distribution at the active site of thiolsubtilisin is similar to that formed during catalysis by the serine enzyme while in the latter the negatively charged tetrahedral adduct forms an ion pair with the imidazolium ion. Thus, comparison of the hydrogen bonds of subtilisin and thiolsubtilisin may provide information about the ground and transition states of the catalytic process. In addition, thiolsubtilisin can be regarded as a model for thiol proteases, e.g., papain. NMR data on the state of ionization of the imidazole can confirm the existence of the mercaptide–imidazolium ion pair in thiolsubtilisin. The existence of this ion pair was proposed on the basis of results inferring that the thiol group exists in its dissociated form (Polgár, 1974). Such an activation mechanism is also of importance in other thiol enzymes (Polgár, 1977).

We report ^{1}H NMR studies that were performed in 80/20 (v/v) $H_2O/^2H_2O$ employing correlation spectroscopy (Dadok & Sprecher, 1974). This technique allowed us to examine the very low field proton in serine proteases that was first reported by Robillard & Shulman (1972, 1974a,b). Those authors, as well as Markley (1978), presented considerable evidence assigning that proton to the catalytic center of these enzymes, specifically between Asp and His ($N^{\delta 1}$-H). We report evidence for a stable hydrogen bond in thiolsubtilisin and in the phenylboronic acid adduct of the serine enzymes but not in the native subtilisins. The results also have direct bearing on the state of ionization of His and Cys at the catalytic site of thiolsubtilisin but not on the native serine enzyme in which the low-field proton is not observed.

Experimental Procedures

Materials. Subtilisins Carlsberg and Novo (the latter equivalent to BPN) were purchased from Sigma, St. Louis, MO, phenylboronic acid was from Aldrich, Metuchen, NJ, and phenylmethanesulfonyl fluoride was from Sigma.

Synthesis of Thiolsubtilisins. Carlsberg and Novo thiolsubtilisins were prepared from the corresponding serine enzymes. The crude preparations were purified on an agarose–mercurial column as described before (Polgár, 1976). The mercury derivatives so obtained were lyophilized and kept at 4 °C until further use. No loss in the thiol content was observed during 1 year of storage as determined by titration of the liberated thiol enzyme with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959).

Carboxamidomethylthiolsubtilisin. The "free" thiol enzyme was regenerated from the mercuric salt of thiolsubtilisin Novo by incubation at room temperature in 15 mM cysteine, pH 7, for 30 min. Next the protein was chromatographed on a Sephadex G-25 column that had been equilibrated with 0.05 M phosphate buffer, pH 7.0. The free thiol enzyme (0.2 mM) was reacted with 2 mM iodoacetamide for 2 h. The alkylated enzyme was next chromatographed on a Sephadex G-25 column that had been equilibrated with 1 mM phosphate and 0.03 M KCl, pH 7.0. The purified alkylated thiol enzyme was lyophilized. Alkylation of thiolsubtilisin was complete according to titration with 5,5′-dithiobis(2-nitrobenzoate).

Preparation of Samples for ^{1}H NMR. The thiol enzyme was liberated from its mercuric derivative by addition of a 2–5 molar excess of mercaptoethanol or dithiothreitol. Regeneration of the thiol enzyme was complete within 1 h under these conditions. The pH of the samples was adjusted initially in 1.7-mL Eppendorf plastic vials by quick addition and mixing in of approximately 0.1 N acid or base. Typically, each sample was prepared in 80/20 (v/v) $H_2O/^2H_2O$ (the ^{2}H needed for lock) containing 0.5 M KCl. Further adjustments of pH were accomplished within the 5-mm sample tubes by employing an Ingold combination electrode.

NMR Measurements. All measurements were performed at low temperature (near 0 °C), and all samples were run within 4 h of initial dissolution in buffers. The measurements were performed at 360 MHz on a Bruker WH-360 spectrometer in the proton correlation mode (Dadok & Sprecher, 1974) at the Middle Atlantic NMR Facility located at the University of Pennsylvania, School of Medicine. Typically, the 1–2 mM solutions required ca. 1000 1-s rapid scan accumulations. It was found that careful application of the base-line correction routine was imperative; alternatively many artifacts resulted. Chemical shifts in Table I and Figures 1–8 are given in parts per million downfield from DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt).

Results and Discussion

Robillard & Shulman (1972, 1974a,b) and Markley (1978) presented evidence on serine proteases, their zymogens, and their inhibited complexes to support the thesis that the very low field ^{1}H NMR resonances observed only in H_2O pertain to the active site histidine. Very likely the observation concerns $N^{\delta 1}$-H that is capable of forming a hydrogen bond with aspartate. For chymotrypsin A_δ the resonance position was found at 17–18 and about 15 ppm downfield from DSS for protonated and neutral histidines, respectively (Robillard & Shulman, 1972).

Table I summarizes our data on subtilisins: (a) the serine enzymes (Carlsberg and Novo); (b) the serine enzymes in the

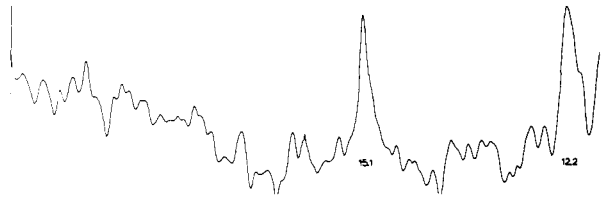


FIGURE 1: Subtilisin Novo, pH 7.1, 360-MHz ^{1}H correlation NMR spectrum recorded at 2 °C (see footnote *d*, Table I). The correlation function was applied to 1016 1-s accumulations. Chemical shifts are in ppm from DSS.

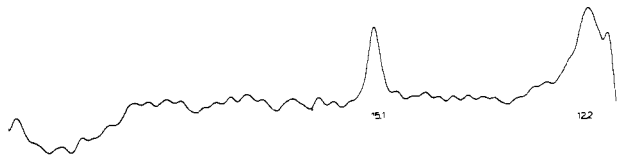


FIGURE 2: Subtilisin Novo, pH 6.05, 360-MHz ^{1}H correlation NMR spectrum recorded at −2 °C (see footnote *d*, Table I). 1000 1-s accumulations were correlated. Chemical shifts are in ppm from DSS.

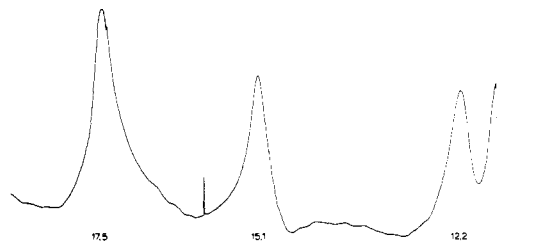


FIGURE 3: Subtilisin Novo, pH 7.5, 2.2 mM + 5 mM phenylboronic acid, 360-MHz ^{1}H correlation NMR spectrum recorded at 2 °C [control experiment for condition 3, Table I (footnote *f*)]. 422 1-s accumulations were correlated. Chemical shifts are in ppm from DSS.

presence of the transition state analogue phenylboronic acid (Matthews et al., 1977, and references cited therein); (c) the thiol enzymes (Carlsberg and Novo) as inactive mercuric salts; (d) the thiol enzymes liberated from their mercuric derivatives by addition of dithiothreitol or mercaptoethanol; and (e) thiol enzyme inactivated by iodoacetamide.

First, it needs to be pointed out that in *all spectra* of *subtilisin* and *thiolsubtilisin* that we have examined there is a pH-independent resonance in H_2O at 15.1 ppm downfield from DSS. This resonance was not apparent in the spectra of subtilisin shown before by Robillard & Shulman (1974a,b). On account of its pH-independent chemical shift, the resonance at 15.1 ppm cannot be assigned to a normal histidine residue. Judging from its broad line width, the 15.1-ppm resonance pertains to a proton on a protein. Impurities in subtilisin preparations would be unlikely to be carried along throughout all synthetic and purification procedures performed on thiolsubtilisin. Concerning the 17.5- and 17.7-ppm resonances, Table I provides the following information: (1) The resonances are not observed at any pH (5.5–9) in either Carlsberg or Novo serine enzymes (Figures 1 and 2) at 1–2 mM concentration at 2 °C (in a limited number of experiments, as low as −2 °C). (2) The 17.5-ppm resonance is observed at *all* pHs (6 to above 8) in both serine enzymes when phenylboronic acid is added as was reported by Robillard & Shulman (1974b) for the Novo enzyme (see Figure 3). (3) If the serine enzyme is first irreversibly inactivated with phenylmethanesulfonyl fluoride, addition of phenylboronic acid no longer induces appearance of the 17.5-ppm resonance (Figure 4). This is consistent with the assignment of the resonance to the catalytic center, since both reagents acylate the catalytic serine. (4) The mercuric derivatives of the thiol enzymes from both sources do not exhibit the low-field 17.7-ppm resonance between pH 5.25 and

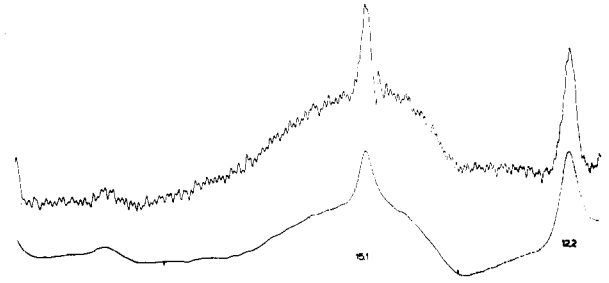


FIGURE 4: Subtilisin Novo, pH 6.6, incubated with 3.3 mM phenylmethanesulfonyl fluoride for 2.5 h and then with 5.5 mM phenylboronic acid added (condition 3 and footnote *f*, Table I) (360-MHz ^{1}H correlation NMR spectrum). 1100 1-s accumulations (jagged curve) were correlated (smooth curve). The spectrum was recorded at 2 °C. Chemical shifts are in ppm from DSS.

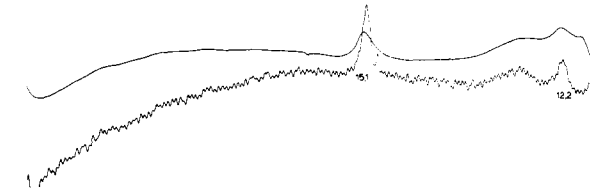


FIGURE 5: Thiolsubtilisin Novo–mercuric derivative, pH 6.67 (footnote *g*, Table I), 360-MHz ^{1}H correlation NMR spectrum recorded at 2 °C. 1100 1-s accumulations (jagged curve) were correlated (smooth curve). Chemical shifts are in ppm from DSS.

FIGURE 6: Thiolsubtilisin Novo, pH 7.3 (footnote *h*, Table I; liberated by employing dithiothreitol), 360-MHz ^{1}H correlation NMR spectrum recorded at 2 °C. 1000 1-s accumulations were correlated. Chemical shifts are in ppm from DSS.

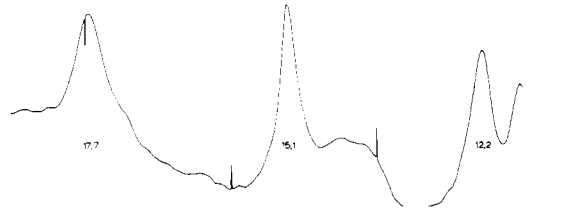


FIGURE 7: Thiolsubtilisin Carlsberg, pH 6.37, 360-MHz ^{1}H correlation NMR spectrum recorded at 2 °C (footnote *h*, Table I; liberated by employing mercaptoethanol); 1400 1-s accumulations were correlated. Chemical shifts are in ppm from DSS.

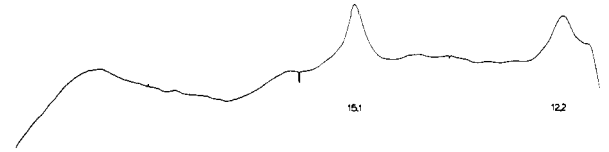


FIGURE 8: Carboxamidomethylthiosubtilisin Novo, pH 6.92, 360-MHz ^{1}H correlation NMR spectrum recorded at 2 °C (footnote *i*, Table I). 1200 1-s accumulations were correlated. Chemical shifts are in ppm from DSS.

7.8 (Figure 5). (5) The thiol enzymes from both sources (Figures 6 and 7) do give rise to the low-field 17.7-ppm resonance (pH 5.38–7.95 clearly, with diminishing intensity from pH 7.95 to 8.37). (6) Carboxamidomethylthiolsubtilisin Novo does not give rise to the low-field 17.7-ppm resonance at pH

Table I: Observation of the Low-Field ^{1}H NMR in Aqueous Solutions of Subtilisins[a]

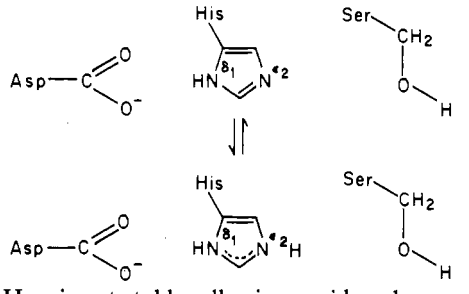
condition	pH[b] (no. of exptl points in pH range)	chemical shift[c] (ppm)
(1) serine enzymes		
subtilisin Novo[d]	5.34–8.73 (12)	none obsd
subtilisin Carlsberg[d]	5.91, 6.59	none obsd
(2) serine enzymes with $C_6H_5B(OH)_2$ added[e]		
subtilisin Novo	5.97, 6.0, 7.0, 7.5, 7.99	17.5
subtilisin Carlsberg	6.71, 7.15, 7.99	17.5
(3) PMSF inactivated[f] subtilisin Novo	6.67	none obsd
$+C_6H_5B(OH)_2$	6.80	none obsd
control for above [no PMSF but $C_6H_5(OH)_2$]	7.50 at 2 °C[j]	17.5
	7.50 at 7 °C[j]	17.5
	7.50 at 12 °C[j]	17.5
(4) thiolsubtilisin Novo		
(a) mercuric salt[g]	6.7, 7.8, 8.3	none obsd
(b) Hg salt + 10 mM[h] dithiothreitol	6.5, 7.3, 7.5, 8.2	17.7
(c) Hg salt + 10 mM[h] mercaptoethanol	7.0, 7.96	17.7
(5) thiolsubtilisin Carlsberg		
(a) mercuric salt[g]	5.25	none obsd
(b) Hg salt + 10 mM[h] mercaptoethanol	5.38–8.37 (7)[k]	17.7
(6) thiolsubtilisin Novo, iodoacetamide inactivated[i]	6.92	none obsd

[a] All spectra were recorded at 360 MHz and 2 ± 1 °C unless otherwise noted, nonspinning, and employing 80/20 (v/v) $H_2O/^2H_2O$. All spectra indicated the presence of other resonances at 15.1 and 12.2 ppm. [b] Measured at room temperature, ±0.05-unit uncertainty. [c] Parts per million (±0.1) downfield from DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt). The sweep width was set up so that no resonances upfield from 12.0 ppm were recorded. The resonances at 15.1 and 12.2 ppm appeared in all spectra and, therefore, are not discussed but are evident in the figures. [d] Concentrations of protein were 1.5–2 mM in 0.5 M KCl, sometimes with 0.05 M $NaHCO_3$ buffer; otherwise the protein provided all the buffering. The pHs represent all values at which spectra were taken. Usually no more than two or three spectra were taken on the same sample. [e] In a typical experiment 3–5 mM $C_6H_5B(OH)_2$ was present in addition to 1.5–2 mM enzyme. The phenylboronic acid was made up as a 0.1 M aqueous solution, and aliquots of this were added to the protein solution. The pH represents that of the total mixture. [f] A 2.2 mM subtilisin Novo sample was prepared. A 0.5-mL portion was made 3.5 mM in phenylmethanesulfonyl fluoride (the latter was prepared as a 10 mg/mL solution in dioxane) and incubated at room temperature for 2.5 h, and the spectrum was collected in the last half hour. Next the solution was made with respect to $C_6H_5B(OH)_2$, and the spectrum was recorded immediately. To the other half of the protein solution was added only $C_6H_5B(OH)_2$, and the spectrum was recorded as usual. On this solution a limited study of the temperature dependence was also performed. [g] 1.8–2 mM protein solutions in 0.5 M KCl and sometimes $NaHCO_3$. [h] Dithiothreitol and mercaptoethanol were first made up as 0.1 M aqueous solutions (within a few hours of use), and aliquots of these were added to make the protein solutions 5–10 mM in these reagents. The protein was incubated from 1 to 3 h at room temperature (22–25 °C) with these reagents prior to recording of the spectrum. [i] Concentration of protein was approximately 1.4 mM. It was prepared as described under Experimental Procedures. [j] The resonances at 15.1 and 17.5 ppm from DSS both decreased in intensity substantially (to less than half compared to that at 12.2 ppm) in this temperature range. [k] Compared to the intensity of the resonance at 15.1 ppm, the intensity of the resonance at 17.7 ppm became progressively smaller between pH 7.97 and 8.37, hardly detectable at the latter pH.

6.92, 2 °C (Figure 8), conditions under which the thiol enzymes do. Conditions 4 and 6 inhibit formation of thiolate anion.

The chemical shifts of the resonances observed at 17.5 ppm in subtilisin–phenylboronic acid complexes and at 17.7 ppm in thiosubtilisin in a wide pH range suggest that the resonance pertains to a nitrogen-bound proton in an imidazolium species (Robillard & Shulman, 1972, 1974a,b). The fact that such a resonance is not observed at any pH in the native subtilisins even at −2 °C is in contrast to some previous results. X-ray diffraction studies indicated that there exists a hydrogen bond between the $N^{\delta 1}$-H of histidine and the adjacent aspartate. The conformation of the crystalline enzyme could be slightly different from that existing in solution. ^{1}H NMR studies by Robillard & Shulman (1974a) reported this low-field resonance in native chymotrypsin A_δ at a variety of pHs and in native trypsin and in α-lytic protease near pH 3. Markley (1978) later confirmed the experiments on trypsin and claimed the existence of a very small peak at pH 7 at 17.8 ppm. This latter observation, however, would be inconsistent with the earlier reported pK_a of 5 (Markley & Porubcan, 1976) for the catalytic histidine. The pK_a of 5 at pH 7 would require a neutral imidazole ring whose $N^{\delta 1}$-H resonance should be at or near 15 ppm, according to Robillard and Shulman's data on chymotrypsin A_δ. Therefore, in native enzymes at pH >4, only in chymotrypsin A_δ has the resonance at 15–18 ppm been clearly observed. Subtilisin undergoes denaturation below pH 5.5; therefore, data could not be obtained at lower pHs. The observation of the very low field resonance in subtilisin Novo at pH 6.0 in the presence of 0.1 M *N*-acetyl-L-tryptophan (Robillard & Shulman, 1974a) is consistent with our results. This compound can bind at the active site, and the negative charge of its carboxyl group can cause an effect similar to that found in the phenylboronic acid adduct or in thiolsubtilisin.

The absence of the low-field resonance in the native subtilisin enzymes has two possible explanations: (1) The hydrogen bond between the carboxylate of Asp and His $N^{\delta 1}$-H does not exist



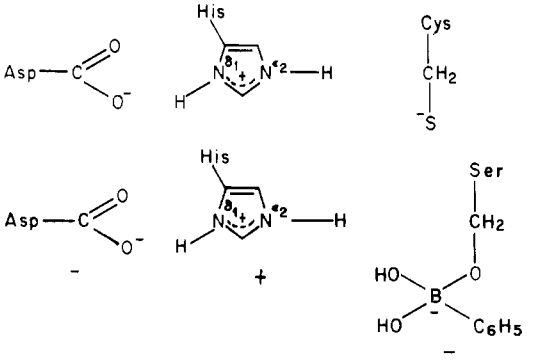
at any pH or is not stable, allowing rapid exchange of $N^{\delta 1}$-H with solvent hydrogens, and this exchange broadening makes observation difficult. Examination of the 15.1- and 17.5-ppm resonances in the subtilisin–phenylboronic acid adduct between 2 and 12 °C and Markley's studies (1978) indicate that the line width of such N-H's is strongly temperature dependent, i.e., exchange broadened. (2) Our observations in subtilisins pertain to $N^{\epsilon 2}$-H whose resonance becomes visible upon hydrogen bonding of $N^{\epsilon 2}$-H to the phenylboronate–Ser adduct or enzyme–CH_2–S^- in thiolsubtilisin. Especially the spectra shown on $N^{\epsilon 2}$-alkylated chymotrypsin A_δ (Robillard & Shulman, 1974b) make the first choice more plausible. Our conclusions below stand, irrespective of which explanation

above is valid for native subtilisins, although we assume for the sake of discussion that the first explanation above is correct.

An important aspect of our results concerns thiolsubtilisins in which the low-field resonance characteristic of the protonated histidine appears. From this observation two conclusions may be drawn: (1) The protonated histidinium ion exists in a wide pH range over which one of us already observed the mercaptide ion form of the thiol group by employing a spectrophotometric technique (Polgár, 1974). Thus the presence of the mercaptide–imidazolium ion pair in thiolsubtilisin is now supported by two totally different experimental approaches, each of which examines a different aspect of this ion pair. (2) During the catalytic process the negatively charged tetrahedral intermediate and the protonated imidazole form an ion pair. Thiolsubtilisins, as well as the phenylboronic acid adducts of the serine enzymes, exhibit similar charge distributions at their active sites. The observation of the low-field resonance in these species but not in native subtilisins suggests that the hydrogen bond between His N^{δ_1}-H and Asp CO_2^- is more stable and likely more important during formation of the tetrahedral intermediate than in the substrate-free state of these enzymes. This is consistent with an earlier proposal (Polgár, 1972) concerning the stabilization of the tetrahedral intermediate by an extended hydrogen-bonding network involving the imidazolium···aspartate hydrogen bond.

According to our results, the hydrogen bond between His N^{δ_1}-H and Asp in subtilisins is very greatly stabilized by the presence of an oxyanion near the N^{ϵ_2}-H. In native subtilisin the lifetime of the hydrogen between N^{δ_1}-H and Asp then must be short on the NMR time scale. Since the line must be broader than ca. 400 Hz, the lifetime must be shorter than 1 ms. The adjacent oxyanion could stabilize the proton between N^{δ_1} and Asp by electronic effects and by restriction of the motion of the imidazole ring. That the latter effect could be quite important is indicated by the rapid temperature-dependent diminution in size of (this study on phenylboronic acid adduct) and disappearance of (Markley, 1978) the low-field resonance. Accordingly, in our opionion, the difference in aqueous solution between native subtilisins and, for example, chymotrypsin A_δ is in the stability of the hydrogen bond rather than its presence or absence. This is a significant difference, not recognized so far, and implies that at a given temperature the imidazole ring (or perhaps aspartate adjacent to it) has more motional freedom in subtilisins than, say, in chymotrypsin A_δ.

Finally, one can conclude from our data that between pH 6 and 8 the proton resides on the imidazole in thiolsubtilisins and in the phenylboronic acid derivatives of the serine enzymes [the chemical shift is at 17.7 and 17.5 ppm, respectively, a position consistent with a protonated histidine in serine proteases; see Robillard & Shulman (1972, 1974a,b) and Markley (1978)]. These results further suggest that in the catalytic intermediate (that bears a similar charge distribution, i.e., − + −), the proton must also reside on the imidazole. Since the proton remains on histidine in the entire pH range studied here, the charge relay is unlikely to be operative during catalysis by subtilisins.



Acknowledgments

Preliminary studies on this problem were performed at the Department of Biochemistry, University of Oxford. Frank Jordan is grateful to R. A. Dwek for his hospitality and advice.

References

Bachovchin, W. W., & Roberts, J. D. (1978) *J. Am. Chem. Soc. 100*, 8041–8047.

Blow, D. M., Birktoft, J. J., & Hartley, B. S. (1969) *Nature (London) 221*, 337–340.

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243–248.

Ellman, G. E. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., & Richards, J. H. (1973) *Biochemistry 12*, 4732–4743.

Kossiakoff, A. A., & Spencer, S. A. (1980) *Nature (London) 288*, 414–416.

Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331–358.

Markley, J. L. (1978) *Biochemistry 17*, 4648–4656.

Markley, J. L., & Porubcan, M. A. (1976) *J. Mol. Biol. 102*, 487–509.

Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., & Kraut, J. (1977) *J. Biol. Chem. 252*, 8875–8883.

Neet, K. E., & Koshland, D. E., Jr. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 1606–1611.

Polgár L. (1972) *Acta Biochim. Biophys. Acad. Sci. Hung. 7*, 29–34.

Polgár, L. (1974) *FEBS Lett. 38*, 187–190.

Polgár, L. (1976) *Acta Biochim. Biophys. Acad. Sci. Hung. 11*, 81–86.

Polgár, L. (1977) *Int. J. Biochem. 8*, 171–176.

Polgár, L., & Bender, M. L. (1966) *J. Am. Chem. Soc. 88*, 3153–3154.

Polgár, L., & Bender, M. L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 1335–1342.

Porubcan, M. A., Westler, W. M., Ibañez, I. B., & Markley, J. L. (1979) *Biochemistry 18*, 4108–4116.

Robillard, G., & Shulman, R. G. (1972) *J. Mol. Biol. 71*, 507–511.

Robillard, G., & Shulman, R. G. (1974a) *J. Mol. Biol. 86*, 519–540.

Robillard, G., & Shulman, R. G. (1974b) *J. Mol. Biol. 86*, 541–558.

Rogers, G. A., & Bruice, T. C. (1974) *J. Am. Chem. Soc. 96*, 2473–2481.