^{1}H NMR Spectroscopic Studies of Selenosubtilisin†

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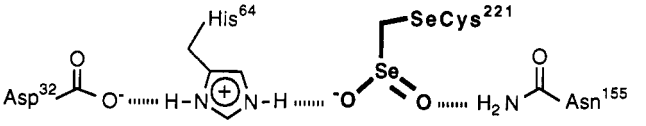
ABSTRACT: Anomalously low-field signals in ^{1}H NMR spectra of serine proteases provide valuable information on the protonation state of the catalytic histidine residue. We have examined the pH dependence of the deshielded protons of three different oxidation states of selenosubtilisin, a semisynthetic selenoenzyme with significant peroxidase activity, in order to evaluate the influence of the selenium prosthetic group on the hydrogen-bonding network in the modified active site. In the spectra of the anionic seleninate and selenolate derivatives, two resonances were observed at 18.0 and 15.5/14.0 ppm, assigned respectively to the Nδ1 and Nϵ2 protons of protonated His64. These signals were apparent from pH 4 to above pH 10, indicating that the negatively charged prosthetic group increases the stability of the imidazolium dramatically, raising its pK_a by at least 3–4 pH units. In contrast, a neutral selenenyl sulfide species exhibits no deshielded proton signals at 18 ppm at any pH but has a weak signal at 14.1 ppm above pH 7 which was assigned to the Nδ1 imidazole proton of neutral His64. While the pK_a of His64 appears normal ($\sim$7) in this derivative, the selenenyl sulfide substitution may alter the orientation of the imidazole ring within the active site for steric reasons. Together with data on the influence of pH on peroxidase activity, these results suggest that selenosubtilisin's His64 acts as a general acid facilitating the reduction of the selenenyl sulfide to selenolate by thiols.

Replacement of the catalytically essential serine in subtilisin with selenocysteine converts the alkaline protease into a peroxidase (Wu & Hilvert, 1990). The engineered selenoenzyme, selenosubtilisin, efficiently catalyzes the reduction of a broad range of alkyl hydroperoxides with concomitant oxidation of the donor substrate 3-carboxy-4-nitrobenzenethiol. This reaction mimics the activity of the important naturally occurring selenoenzyme glutathione peroxidase [EC 1.11.1.9] which protects mammalian cells against oxidative damage caused by hydroperoxides and oxygen-centered radicals (Ladenstein, 1984; Wendel, 1980). As in the case of glutathione peroxidase, a kinetic mechanism has been proposed for selenosubtilisin that involves interconversion of three derivatives of the selenium prosthetic group: a selenol (ESeH), a selenenic acid(ESeOH), and a selenenyl sulfide (ESeSAr) (Wu & Hilvert, 1990). Consistent with this proposal, ping-pong kinetics have been observed under steady-state conditions, and the selenolate and selenenyl sulfide forms of the enzyme have been isolated and shown to be competent as catalysts. The latter two species have been characterized by ^{77}Se NMR[1] spectroscopy (House et al., 1992), as has a seleninic acid species ($ESeO_2H$) that is not believed to be part of the normal catalytic cycle but may become important at high peroxide concentrations. The spectroscopic analysis not only confirmed the chemical identity of the various selenium derivatives but also revealed that the enzyme-bound selenol and seleninic acid have unusually low pK_a values compared to typical selenium compounds and are deprotonated at all accessible pH's. The 2.0-Å structure of the oxidized enzyme showed that stabilization of the conjugate bases of these species can be attributed to specific hydrogen-bonding and electrostatic interactions within the active site, particularly with the side chains of His64 and Asn155 as illustrated in **1** (Syed et al., 1993).



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In native subtilisin, Asp32 and His64 are part of the catalytic triad that is essential for proteolytic activity (Kraut, 1977; Wells & Estell, 1988). The carboxylate side chain of Asp32 forms a hydrogen bond to Nδ1 of His64, which orients the imidazole ring within the active site and allows it to serve as a general base in catalysis. The side chain amide of Asn155, together with the main chain amide of residue 221, constitutes the oxyanion hole that helps to stabilize the anionic oxygen in the tetrahedral transition states and intermediates that form as the enzyme is transiently acylated at Ser221. It is of interest to know whether and how these same groups participate in the peroxidase reaction promoted by selenosubtilisin. Preliminary pH-rate data on the reduction of *tert*-butyl hydroperoxide by 3-carboxy-4-nitrobenzenethiol, for example, indicate that protonation of an enzymic group with a pK_a of about 7 is required for optimal activity (Bell et al., 1992). The ionizing

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DIFP, diisopropyl fluorophosphate; PBA, phenylboronic acid; PNPA, *p*-nitrophenyl acetate; PMS-, phenylmethanesulfonyl; DIP-, diisopropylphosphoryl; MIP-, monoisopropylphosphoryl; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; DTT, dithiothreitol; NMR, nuclear magnetic resonance; Sec221, selenocysteine 221; S/N, signal/noise.

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residue is presumably His64, which has a pK_a of 7.22 in the unmodified protein (Jordan et al., 1985). Although the pH-rate profile suggests that this residue is acting as a general acid in selenosubtilisn rather than as a general base, more data are clearly required to define its precise role in catalysis.

^{1}H NMR spectroscopy has proved to be a powerful tool for studying the structural and chemical properties of the active sites of serine proteases. For example, Robillard and Shulman (1972, 1974a,b) demonstrated some years ago that anomalously low-field resonances in the spectra of chymotrypsin [i.e., 15–18 ppm from 4,4-dimethyl-4-silapentane-1-sulfonate (DSS)] could be attributed to the N–H imidazole protons of the active site histidine residue. Their original findings have been verified with many other serine proteases and their assignments confirmed using a specifically ^{15}N-labeled protease and ^{15}N NMR spectroscopy (Bachovchin, 1985). Because the chemical shifts of the imidazole protons are sensitive both to the ionization state of the histidine residue and to chemical modification of the catalytically essential serine, this technique provides valuable information about pK_a values and hydrogen-bonding patterns within the active site. In the present work we have utilized 500-MHz ^{1}H NMR spectroscopy to examine the effects of different selenium oxidation states on the properties of His64 at the catalytic center of selenosubtilisin.

MATERIALS AND METHODS

Subtilisin Carlsberg and subtilisin BPN′ were purchased from Sigma Chemical Co. Phenylmethanesulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DIFP), phenylboronic acid (PBA), and *p*-nitrophenyl acetate (PNPA) were purchased from Aldrich Chemical Co. Subtilisin activity was assayed spectrophotometrically via hydrolysis of PNPA (Kezdy & Bender, 1962).

PMS-Subtilisins. Subtilisin was sulfonylated by adding 250 μL of PMSF (20 mg/mL in acetonitrile) to a solution of the enzyme (120 mg of the Carlsberg or BPN′ form) in 10 mM PIPES buffer, pH 7. Following a 30-min incubation, the modified protein was isolated by size exclusion chromatography (Sephadex G-15 equilibrated in H_2O) and lyophilized.

Selenosubtilisin. Selenosubtilisin was prepared from subtilisin Carlsberg according to the method of Wu and Hilvert (1989). In a typical experiment, PMS-subtilisin (1.2 mM) and HSe^- (8.5 mM) were allowed to react in 15 mL of PIPES buffer (10 mM, pH 7.0) containing 10 mM $CaCl_2$ for 40 h at 40 °C. Yields of purified selenosubtilisin were 30–40% based on PMS-subtilisin.

The seleninic acid form of selenosubtilisin ($ESeO_2H$) was prepared by sequentially dialyzing the protein against 20 mM dithiothreitol (DTT), 20 mM H_2O_2, and then exhaustively against buffer alone (10 mM PIPES, 10 mM $CaCl_2$, pH 7.0). Reaction of the resulting oxidized enzyme with 3 equiv of 3-carboxy-4-nitrobenzenethiol at pH 5.0 for 15 min at ambient temperature yielded the corresponding selenenyl sulfide derivative (ESeSAr) (Wu & Hilvert, 1990). The fully reduced enzyme (ESeH) was prepared by treating either $ESeO_2H$ or ESeSAr with 30 mM DTT under an inert atmosphere. Prior to NMR spectral analysis, all samples were dialyzed in deionized, distilled water, lyophilized, and stored at 0 °C.

DIP- and MIP-Subtilisin. Subtilisin was site-selectively phosphorylated by addition of a 10-fold excess of DIFP in 30 μL of isopropanol to a 1-mL solution of the enzyme (3.7 mM) in 10 mM PIPES buffer (pH 7). The reaction was allowed to stir overnight to ensure complete hydrolysis of unreacted reagent. The resulting DIP-enzyme was purified via size-exclusion chromatography (Sephadex G-15 equilibrated in H_2O) and lyophilized for NMR spectral analysis. Alternatively, DIP-subtilisin was "aged" by dialysis against H_2O at 36 °C to produce the monoisopropylphosphoryl adduct, MIP-subtilisin (van der Drift et al., 1985).

Table I: Summary of the Low-Field ^{1}H Resonances Observed for Various Subtilisin and Selenosubtilisin Derivatives

derivative	chemical shift δ (ppm)		pH
$ESeO_2^-$	18.0	15.5	(<9)
ESe^-	18.0	14.0	(<9)
ESeSAr		14.1	(>7)
ESeSAr			(<7)
E-PBA	17.6	15.0	(<9)
E-MIP	17.8	14.0	(<9)

Buffers and pH Measurements. All NMR experiments were conducted in sodium phosphate buffer (10 mM) in 20:80 D_2O/H_2O (v/v) with pH measured before and after NMR spectral analysis. Sodium carbonate (10 mM) was added to high pH (>9.0) buffers in order to maintain buffering capacity and constant pH. The pH of all samples was adjusted by addition of 0.1 M NaOH or 1.7 M acetic acid and was measured at the corresponding probe temperature for each experiment. Measurements of pH were made by using a Microelectrode MI-410 probe coupled to an Orion SA 720 pH meter.

Instrumental. ^{1}H NMR spectra were acquired on a Bruker AM500 operating with a 16 bit digitizer. A soft pulse of duration 125.5 ms was positioned at 21 ppm. The carrier position and pulse width were chosen to maximize signals in the vicinity of 14–18 ppm and to minimize the water peak at 5.04 ppm. The 125.5-ms pulse width provided reasonably uniform ($\geq 0.80M_o$) coverage for resonances within ±10 ppm (5 kHz) of the carrier. The probe temperature was set at 4.0 ± 0.1 °C, and DSS was used as an external chemical shift standard. Typically an average of 2048 FID's were acquired, with protein concentrations ranging from 1 to 2 mM. A line broadening of 10 Hz was applied to each FID before Fourier transformation.

RESULTS

Selenosubtilisin. Representative 500-MHz ^{1}H NMR spectra of selenosubtilisin are illustrated in Figure 1 and the relevant data summarized in Table I. At low pH, the seleninate form of the enzyme ($ESeO_2^-$) exhibits two low-field resonances of equal intensity at 18.0 and 15.5 ppm (Figure 1A). The deshielded proton that resonates at 15.5 ppm titrates above pH 8, while that at 18.0 ppm disappears above pH 10. Above pH 10 no signals are detected between 13 and 19 ppm. The low-field region of the ^{1}H NMR spectrum of the selenolate form of selenosubtilisin (ESe^-) (Figure 1B) closely resembles that of the seleninate derivative, with two resonances at 18.0 and 14.0 ppm. Again, the signals are observed from pH 4.3 to 7.3 but gradually broaden into the baseline as the pH is raised further; no highly deshielded protons are detected above pH 10.

Figure 1C shows the ^{1}H NMR spectra obtained with the selenenyl sulfide derivative of selenosubtilisin formed upon reduction of the oxidized enzyme with 3-carboxy-4-nitrobenzenethiol. At low pH (≤7) no signals are observed in the region 13–19 ppm. As the pH is raised, however, a relatively weak resonance appears at 14.1 ppm. The chemical shift of this new signal is similar to that observed for the imidazole Nδ1–H proton on the neutral form of the catalytic histidine in chymotrypsin (Robillard & Shulman, 1972, 1974a).

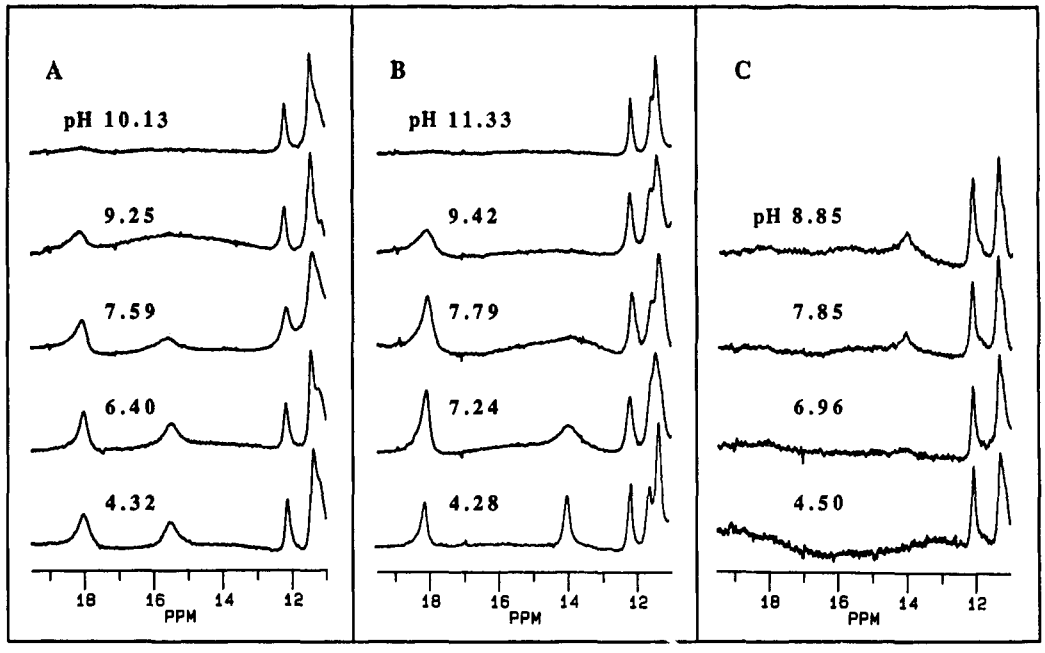

FIGURE 1: Representative 500-MHz ^{1}H NMR spectra of three oxidation states of selenosubtilisin over the pH range 4–11.5: (A) oxidized selenosubtilisin ($ESeO_2^-$); (B) the selenolate form of selenosubtilisin (ESe^-); and (C) the selenenyl 3-carboxy-4-nitrophenylsulfide form of selenosubtilisin (ESeSAr).

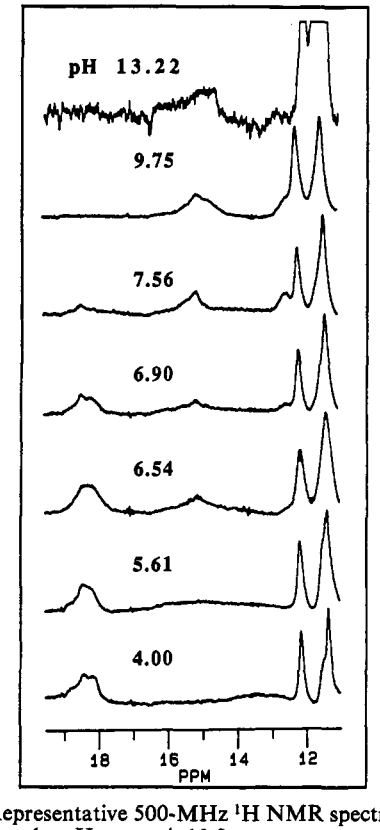

FIGURE 2: Representative 500-MHz ^{1}H NMR spectra of subtilisin Carlsberg over the pH range 4–13.2.

Subtilisin and Derivatives. To place the selenosubtilisin data in perspective, the 500-MHz ^{1}H NMR spectrum of native subtilisin Carlsberg was reexamined under conditions identical to those reported in Figure 1. The data are presented in Figure 2. At pH 4.0 overlapping resonances are apparent at 18.1 and 18.4 ppm. In previous studies these signals were not resolved and had been assigned to the Nδ1 proton of the imidazolium form of His64 which is involved in a hydrogen bond with Asp32. Circular dichroism measurements on the sample confirmed the conformational integrity of the enzyme under these conditions. As the pH is raised, these signals gradually disappear into the baseline and a set of resonances centered at 15.2 ppm appears. The pH titration is completely reversible, and the new signals, corresponding to the N–H protons on the neutral imidazole of His64, are observed up to pH 13.2, although the S/N ratio is poor at very high pH. Qualitatively similar results were obtained with subtilisin BPN′ (data not shown). Our results are thus in accord with the findings of Robillard and Shulman (1974a,b) and with recent work by Zhong and Jordan (F. Jordan, personal communication). They differ from data reported by Jordan and Polgár (1981), who did not detect a signal at 18 ppm for native subtilisin at any pH, but observed instead a pH-independent resonance at ~15 ppm. While the origins of this discrepancy are unknown, given the similarity of Jordan and Polgár's spectra to our high pH data, it is possible that their samples were considerably more basic than reported.

Chemical modification of the catalytically essential serine residue in serine proteases is known to affect the chemical shifts of the low-field histidine resonances due to changes in the hydrogen-bonding patterns within the active site (Robillard & Shulman, 1974a,b). As the ^{1}H NMR spectra of selenosubtilisin differ markedly from those of unmodified subtilisin, several derivatives of the protease were examined under our experimental conditions. The derivatives were chosen to evaluate the effect of modifying Ser221 with both anionic and neutral substituents on the hydrogen-bonding interactions of His64.

Boronic acids are competitive inhibitors of serine proteases such as subtilisin, forming covalent tetrahedral adducts with Ser221. The boronate derivative resembles the presumed transition state for hydrolysis of an acyl enzyme intermediate, and structural studies have shown that the boronate oxygens are hydrogen bonded both to His64 and to the oxyanion hole formed by the amide side chain of Asn155 and the backbone amide of Ser221 (Matthews et al., 1975). The ^{1}H NMR spectra of Carlsberg subtilisin in the presence of a 2.5-fold excess of phenylboronic acid are shown in Figure 3A. Below pH 5.0, only broad resonances attributable to the unmodified protein are seen, consistent with the previous report that

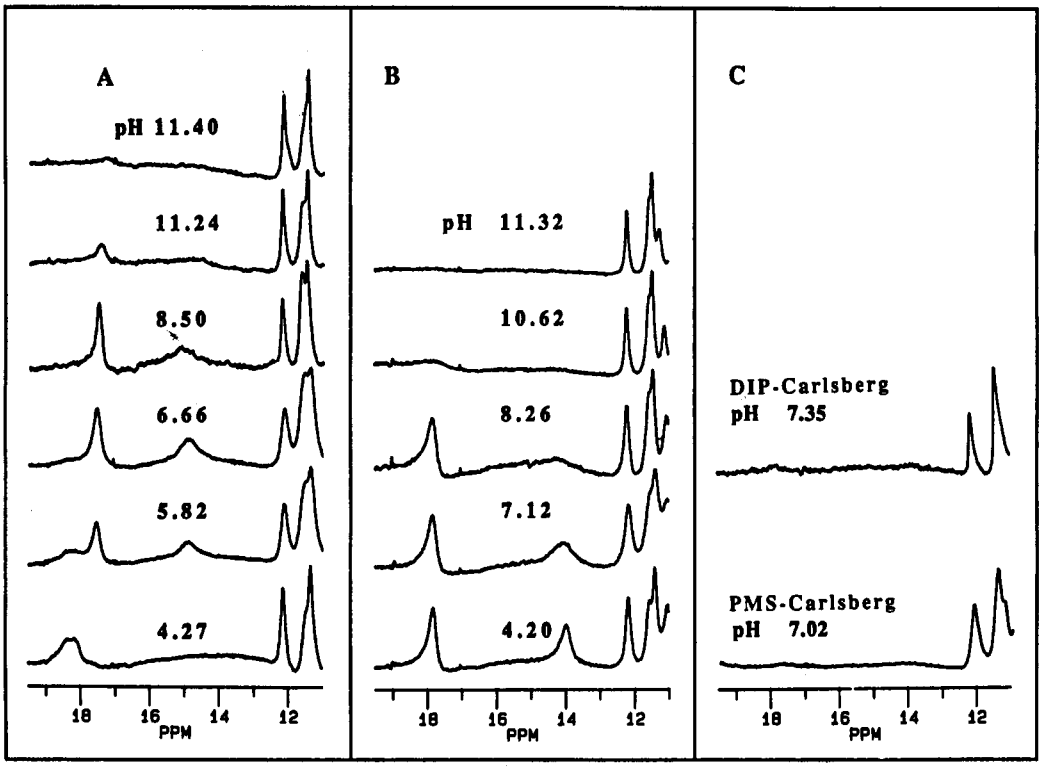

FIGURE 3: (A) 500-MHz ^{1}H NMR spectra of subtilisin Carlsberg (2 mM) inhibited by phenylboronic acid (5 mM) over the pH range 4.3–11.4; (B) 500-MHz ^{1}H NMR spectra of MIP-subtilisin Carlsberg over the pH range 4.2–11.3; (C) 500-MHz ^{1}H NMR spectra of PMS- and DIP-subtilisin Carlsberg. For clarity, not all of the spectra that were acquired are shown.

phenylboronic acid binds poorly to the enzyme at low pH (Philipp & Bender, 1971). As the pH is raised, these resonances are replaced by two new signals at 17.6 and 15.0 ppm, reflecting formation of the boronate adduct (K_i = 0.23 mM). The latter persist to pH 11.0, at which point titration of the more shielded proton begins. Above pH 11.4, the signal at 17.6 ppm also disappears. The monoisopropylphosphoryl adduct of subtilisin Ser221 is another example of an anionic transition-state analog (van der Drift et al., 1985). A ^{1}H NMR spectral titration reveals two resonances at 14.0 and 17.8 ppm below pH 8.3 (Figure 3B). The 14.0 ppm resonance is absent above pH 8.3, and the resonance at 17.8 ppm broadens with increasing pH, disappearing completely above pH 10.6.

Phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate are potent irreversible inactivators of serine proteases. They react selectively with the nucleophilic serine in the active site to produce neutral tetrahedral adducts. ^{1}H NMR analysis of these derivatives thus complements studies of the negatively charged PBA and MIP derivatives. The PMS and DIP derivatives of Carlsberg subtilisin exhibit no low-field resonances in the 13–19 ppm range at any pH examined (Figure 3C).

DISCUSSION

The low-field region of the selenosubtilisin spectra can be assigned with some confidence by comparison with data for wild-type subtilisin and its derivatives. Serine proteases typically show a single low-field resonance (14–18 ppm) in aqueous solution at low temperature (Robillard & Shulman, 1972, 1974a,b; Markley, 1978). The signal has been attributed to the proton attached to Nδ1 of the active site histidine which participates in a hydrogen bond with the catalytic aspartate, and this assignment was established unequivocally for α-lytic protease by the observation of ^{15}Nδ1–H spin–spin splitting in a sample that had been specifically ^{15}N-labeled at the unique histidine (Bachovchin, 1985).

Consistent with these results, we observed a signal at ~18.3 ppm for wild-type Carlsberg subtilisin at low pH (Figure 2). This signal corresponds to a single proton, as judged by comparison with the sharp and nearly invariant resonance at 12.2 ppm, and titrates to 15.2 ppm as the pH is raised. Analysis of the ratio of protonated to deprotonated species in solution as a function of pH gives an approximate pK value of 7.2 for the ionization, in excellent agreement with the reported pK_a of 7.22 for His64 (Jordan et al., 1985). At 500 MHz, the Nδ1–H resonance for the protonated (18.3 ppm) and deprotonated (15.2 ppm) imidazole ring is actually a poorly resolved multiplet, indicating that the histidine side chain adopts more than one conformation within the active site. A similar conclusion was reached independently from an analysis of the imidazole C–2H region of the subtilisin spectrum (Bycroft & Fersht, 1988).

In contrast to native subtilisin, the anionic seleninate and selenolate derivatives of selenosubtilisin exhibit *two* highly deshielded resonances over a wide pH range. We believe that the signals at 18 ppm and 15.5/14.0 ppm correspond to the protons attached to Nδ1 and Nϵ2 of protonated His64, respectively. These protons are involved in the hydrogen bonds that the imidazolium ring makes with both Asp32 (Nδ1–H) and the seleninate/selenolate prosthetic group (Nϵ2–H) (Syed et al., 1993). The formation of strong hydrogen bonds would account for the slow exchange rates of the respective protons and permit their detection by NMR spectroscopy. The relatively sharp and symmetrical peak shape of the signals indicates that the hydrogen-bonding interactions lock the histidine into a single, well-defined conformation within the modified active site. Furthermore, the persistence of the low-field resonances to very high pH shows that His64's pK_a is significantly raised by its interaction with an adjacent anion.

Our observations with selenosubtilisin have a precedent in the work of Bachovchin and co-workers (1988), who detected both imidazolium protons in the 400-MHz ^{1}H NMR spectrum

of α-lytic protease inhibited by peptide boronic acids. Formation of a tetrahedral boronate complex with the active site serine yields a charge distribution within the modified binding pocket that is similar to that of the anionic selenosubtilisin derivatives. The two low-field signals were unambiguously assigned to the Nδ1 and Nε2 protons of the catalytic imidazolium on the basis of ^{15}N–^{1}H coupling. Because the histidine remains protonated up to pH 10, strong hydrogen bonds to the adjacent aspartate and to the anionic boronate adduct were inferred (Bachovchin et al., 1988) and subsequently verified by X-ray crystallography (Bone et al., 1991). We obtained similar results with subtilisin Carlsberg which had been modified covalently at Ser 221 with either phenylboronic acid or diisopropylphosphate (Figure 3A,B). Two low-field signals were detected in these derivatives below pH 8.5; the more deshielded proton's resonance (δ ~18 ppm) does not disappear until the pH is raised above 10. The crystal structure of the subtilisin–PBA adduct confirms that the protonated His64 is sandwiched betweem the carboxylate side chain of Asp32 and the tetrahedral boronate, making hydrogen bonds to both (Matthews et al., 1975). This arrangement accounts for the observed stabilization of the imidazolium cation and causes its pK_a to be increased by several pH units (Adebodun & Jordan, 1989).

In the complex between α-lytic protease and peptide boronic acids, the proton in the His–Asp hydrogen bond is more shielded than the proton involved in the hydrogen bond between His64 and the boronate (Bachovchin et al., 1988). However, the reverse appears to be true for the anionic subtilisin and selenosubtilisin derivatives we examined. The spectra of each of these exhibit a peak at ~18 ppm, somwhat more upfield than the resonance observed for the Nδ1 proton of protonated His64 in unmodified subtilisin. Since the His–Asp hydrogen bond is common to all four derivatives, assignment of the 18 ppm resonance to the shared proton seems reasonable. The chemical shift of the Nδ1–H signal is known to be sensitive to chemical modification of Ser221 (Robillard & Shulman, 1974a,b), but as direct interactions are precluded, the effect is generally small (<0.4 ppm). Moreover, the magnitude and direction of the chemical shift relative to the unmodified protein is expected to be similar for all anions, including the seleninate and selenolate forms of selenosubtilisin and the boronate and monoisopropylphosphoryl derivatives of native subtilisin, as observed experimentally.

Because the proton attached to Nε2 of protonated His64 is physically near the selenocysteine side chain (Sec221) (Syed et al., 1993), its chemical shift and exchange rate would be expected to be highly sensitive to substitutions at this position. By this criterion, the more shielded of the two low-field signals in the spectra of the anionic (seleno)subtilisin derivatives (δ 14–15.5 ppm) can be assigned to the imidazolium Nε2–H. The chemical shift of this proton will depend not only on the oxidation state and charge of its hydrogen-bonding partner but also on the length and geometry of the bond that can be formed. We note in this context that the seleninate–imidazolium hydrogen bond appears to be stronger than that of the selenolate–imidazolium ion pair, given the more downfield position of the proton resonance (e.g., 15.5 vs 14.0 ppm).

The relative exchange rates of the Nε2 and Nδ1 protons provide additional support for the proposed assignments. The signal at 14–15.5 ppm undergoes exchange broadening at substantially lower pH's than the 18 ppm signal, consistent with the hydrogen bond between His64 and the anionic seleninate or selenolate (and between His64 and the boronate or phosphate adducts of Ser221) being considerably more solvent assessible than the buried His–Asp hydrogen bond.

Although the imidazolium form of His64 is greatly stabilized through proximity to the seleninate and selenolate moieties of selenosubtilisin, it was not possible to determine an accurate pK_a for this species. Signals for the corresponding neutral imidazole were never observed in these derivatives, even at very high pH's. Nevertheless, His64's pK_a must be at least 3–4 pH units higher than in native subtilisin, since substantial amounts of the imidazolium species are detected in the ^{1}H NMR spectra above pH 10. These findings complement results from an earlier ^{77}Se NMR study supporting the existence of seleninate–imidazolium and selenolate–imidazolium ion pairs at the enzyme active site (House et al., 1992). The latter is analogous to the thiolate–imidazolium ion pair previously detected at the catalytic center of thiolsubtilisin, a related semisynthetic enzyme (Jordan & Polgár, 1981).

The high pK_a value of His64 in the $ESeO_2^-$ and ESe^- forms of selenosubtilisin makes it unlikely that ionization of histidine is kinetically important either for reduction of the seleninate by thiol or for oxidation of the selenolate by peroxide during catalysis. Recent kinetic studies support this conclusion. For example, reduction of the enzyme-bound seleninate with 3-carboxy-4-nitrobenzenethiol is acid catalyzed, but no break was observed in the pH-rate profile between pH 4.5 and 9 (Bell et al., 1992). In addition, although ionization of a group with an apparent pK of ~7 was detected in the selenosubtilisin-catalyzed peroxidase reaction under turnover conditions, this value is too low to reflect deprotonation of the selenolate–imidazolium ion pair (Bell et al., 1993).

The selenenyl sulfide derivative of selenosubtilisin, formed upon reduction of the seleninate with 3 equiv of 3-carboxy-4-nitrobenzenethiol, is believed to be a key intermediate in the peroxidase reaction (Wu & Hilvert, 1990). This species should also have a very different active site charge distribution than the anionic seleninate or selenolate derivatives. Although ESeSAr's aromatic substituent bears a negatively charged carboxylate, this group must perforce bind some distance from the active site histidine, which is likely to be found closer to the neutral selenenyl sulfide linkage. Indeed, the ESeSAr spectra (Figure 1C) differ strikingly from those of the anionic species (Figure 1A,B). They are more similar to the spectra of the neutral PMS- and DIP-subtilisin adducts [Figure 3C; see also Bachovin (1986)] in that no low-field resonances are observed between 13 and 19 ppm at low pH. Although the His–Asp bond is probably not broken in these derivatives (Wright et al., 1969; Matthews et al., 1977), neutral and bulky substituents at residue 221 apparently fail to lock His64's side chain into place. They may, in fact, induce subtle conformational changes within the active site that facilitate rapid solvent exchange of the nitrogen-bound imidazolium protons. By increasing the exchange rate sufficiently, relative to the NMR time scale, the highly deshielded protons would become experimentally undetectable.

As the pH of the selenenyl sulfide sample is raised above 7.5, a new resonance appears at 14.1 ppm (Figure 1C). This signal presumably corresponds to the Nδ1 imidazole proton of His64, although the His–Asp hydrogen bond appears weaker than usual as judged by the low-intensity and relatively shielded position of the resonance. In contrast, the Nδ1 proton is not detected for the neutral PMS- and DIP-subtilisin derivatives at any pH. We believe that the active site histidine in the selenenyl sulfide derivative titrates normally, i.e., with a pK_a of ~7, given the similar pH dependence of its imidazole Nδ1 proton and that of unmodified subtilisin Carlsberg (compare Figure 1C with Figure 2). If this conclusion is correct, then

histidine ionization presumably accounts for the inflection observed in the pH-rate profile of the selenosubtilisin-promoted peroxidase reaction. Our results would further suggest that reduction of the selenenyl sulfide by thiolate to give the selenolate plus disulfide is the pH-sensitive step that is monitored in the kinetic experiments. In this regard, the relative weakness of the His–Asp hydrogen bond may have functional significance. Additional work to confirm these hypotheses will require direct determination of His64's pK_a in this and the other selenosubtilisin derivatives. To that end, analysis of the pH dependence of the C–2H proton signals may prove efficacious (Jordan et al., 1985; Bycroft & Fersht, 1988).

Recent advances in FT-NMR instrumentation and magnets have greatly increased the power and versatility of NMR spectroscopy as a tool for investigating biological systems. In the present work, these developments have impacted favorably on our ability to acquire high quality spectra with improved resolution and excellent signal-to-noise ratios. Study of highly deshielded imidazole/imidazolium protons at the catalytic center of selenosubtilisin by ^{1}H NMR spectroscopy has provided valuable information on the protonation state, hydrogen-bonding properties, and catalytic role of His64, underscoring the general utility of this simple technique for characterizing the active sites of chemically or genetically modified serine proteases.

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