Assignment of the $N^{\epsilon 2}H$ and $N^{\delta 1}H$ Resonances at the Active-Center Histidine in Chymotrypsin and Subtilisin Complexed to Peptideboronic Acids without Specific ^{15}N Labeling[1]

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Abstract: A combination of ^{1}H and ^{15}N nuclear magnetic resonance experiments have been carried out to assign the two high-frequency ^{1}H resonances that result from the complexation of subtilisin E and MeoSuc-Ala-Ala-Pro-boroPhe, a potent peptideboronic acid inhibitor of both subtilisins from a variety of sources and chymotrypsin. First, it was demonstrated unequivocally using two auxotrophs of *Bacillus subtilis* that the proton resonances at 16 and 17 ppm pertain to a histidine residue. Next it was shown by both 1D and 2D methods that the two proton resonances pertain to the *same histidine*. Finally, in the subtilisin E-peptideboronate complex, all of the imidazole proton and nitrogen resonances pertinent to this His64 were assigned as follows: $N^{\epsilon 2}$ at 183 and $N^{\delta 1}$ at 189 ppm; $N^{\epsilon 2}H$ at 16 and $N^{\delta 1}H$ at 17.4 ppm; $C^{\epsilon 1}H$ at 9.20 and $C^{\delta 2}H$ at 7.09 ppm. Using the 1D NOE method demonstrated on the subtilisin−peptideboronate complex, the resonances due to complexation were also assigned in the chymotrypsin−peptideboronate complex. The assignments of the two high-frequency resonances are reversed from those assumed in a previous paper from the current authors (Zhong, S.; Haghjoo, K.; Kettner, C.; Jordan, F. *J. Am. Chem. Soc*. **1995,** *117*, 7047−7055), in which the assignments were adopted from the relative chemical shifts assigned on α-lytic protease [Bachovchin, W. W.; Wong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988,** *27*, 7689−7697].

Introduction

Observation of proton resonances corresponding to the mobile nitrogen-bound protons on the imidazole ring of histidines in proteins has been greatly facilitated recently by both hardware and software innovations. While the first such studies date back to the early 1970s,[2,3] only few additional studies appeared for about 15 years.[4−6] One of the chronic problems in such studies is the poor baseline in ^{1}H FT-NMR experiments in the 13−20 ppm range (recorded in mostly aqueous media, typically using binomial water suppression methods). An unequivocal assignment of these resonances to the active center histidine had only been carried out for the serine protease α-lytic protease,[6] in which the sole histidine is located at its active center. Markley and co-workers had shown that the $N^{\epsilon 2}H$ and $N^{\delta 1}H$ resonances could be assigned on a protease inhibitor of modest size in DMSO-d_6 by correlation with the $C^{\delta 2}H$ and $C^{\epsilon 1}H$ resonances, using either 1D or 2D methods.[7] More recently, of the two resonances observed in acidic solution of chymotrypsinogen, the one at 13 ppm was assigned to $N^{\epsilon 2}H$, the one at 18 ppm to $N^{\delta 1}H$ using NOE methods,[8] without the benefit of ^{15}N labeling. In the presence of some potent peptideboronic acid and peptide-related boronic acid inhibitors (so-called transition-state analogues), two resonances can be readily identified in α-lytic protease,[9] trypsin,[10] chymotrypsin,[11,12] and subtilisin.[11] On α-lytic protease, ^{15}N labeling was used to assign the resonances at 16.0 and 16.5 ppm to the $N^{\delta 1}H$ and $N^{\epsilon 2}H$, respectively.[9] Our initial studies on peptideboronic acids centered on the development of methods to observe the boron nucleus (^{11}B), the results of which clearly signaled whether the boron is bound on the enzyme in a trigonal or tetrahedral environment.[13] In the proton spectra of these complexes, the observation of two resonances, similar (though not identical) in chemical shifts to those reported

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(1) Abbreviations: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; NOE, nuclear Overhauser effect; HMQC, heteronuclear multiple quantum correlation; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TPPI, time proportional phase incrementation; BoroPhe, MeoSuc-Ala-Ala-Pro-boroPhe in which the carboxyl terminal carboxylate is replaced by $-B(OH)_2$.

(2) Patel, D.; Woodward, C.; Bovey, F. *Proc. Natl. Acad. Sci. U.S.A.* **1972,** *69*, 599−602.

(3) (a) Robillard, G.; Schulman, R. G. *J. Mol. Biol.* **1972,** *71*, 507−511. (b) Robillard, G.; Schulman, R. G. *J. Mol. Biol.* **1974,** *86*, 541−548. (c) *Ibid*. **1974**, *86*, 519−540.

(4) (a) Markley, J. L. *Biochemistry* **1978** *17*, 4648−4656. (b) Markley, J. L. In *Biological Applications of Magnetic Resonance*; Shulman, R. G., Ed.; Academic Press: New York, 1979; pp 397−461.

(5) Jordan, F.; Polgár, L. *Biochemistry* **1981,** *20*, 6366−6370.

(6) (a) Bachovchin, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1985,** *82*, 7948−7951. (b) Bachovchin, W. W. *Biochemistry* **1986,** *25*, 7751−7759.

(7) Wu, X.; Westler, W. W.; Markley, J. L. *J. Magn. Reson.* **1984,** *59*, 524−529.

(8) Markley, J. L.; Westler, W. W. *Biochemistry* **1996**, *35*, 11092−11097.

(9) Bachovchin, W. W.; Wong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689−7697.

(10) Tsilikounas, E.; Kettner, C. A.; Bachovchin, W. W. *Biochemistry* **1992**, *31*, 12839−12846.

(11) (a) Zhong, S.; Haghjoo, K.; Kettner, C.; Jordan, F. *J. Am. Chem. Soc*. **1995,** *117*, 7048−7055. (b) Zhong, S. Ph.D. Thesis, Department of Chemistry, Rutgers, Newark, NJ, 1991. (c) Haghjoo, K. Ph.D. Thesis, Department of Chemistry, Rutgers, Newark, NJ, 1996.

(12) Sylvia, L. A.; Gerig, J. T. *Biochim. Biophys. Acta* **1995,** *1252*, 225−232.

(13) (a) Adebodun, F.; Jordan, F. *J. Am. Chem. Soc.* **1988**, *110*, 309−310. (b) Adebodun, F.; Jordan, F. *J. Cell. Biochem.* **1989,** *40*, 249−260. (c) Zhong, S.; Jordan, F.; Kettner, C.; Polgar, L. *J. Am. Chem. Soc.* **1991**, *113*, 9429−9435.

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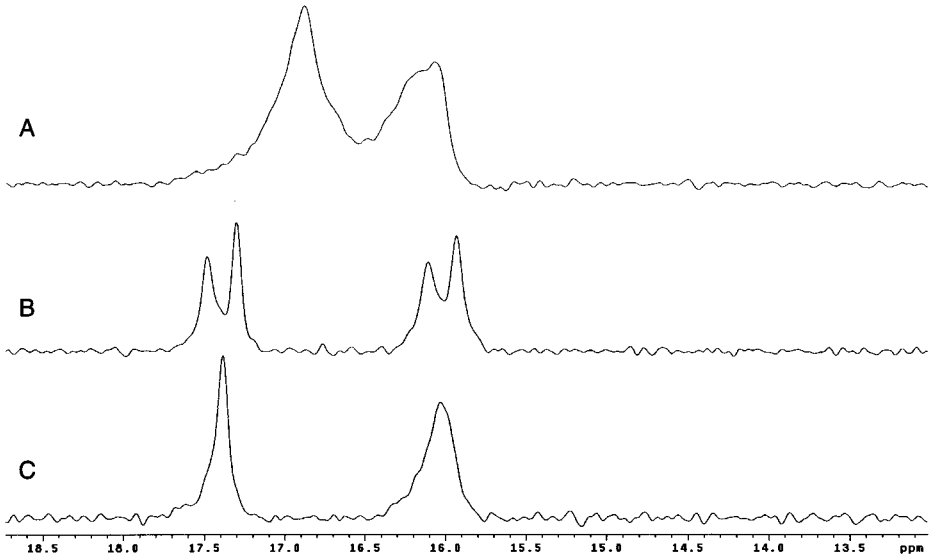
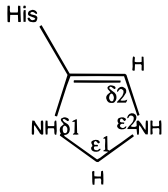


Figure 1. 500-MHz ^{1}H NMR spectra of BoroPhe-inhibited serine proteases at high frequency in H_2O. All spectra measured at pH 6.5 and 25 °C: (A) α-chymotrypsin; (B) ^{15}N-labeled subtilisin E without decoupling; (C) ^{15}N-labeled subtilisin E with ^{15}N decoupling.

for α-lytic protease, prompted us to adopt the relative assignments made on that enzyme, i.e. the $N^{\epsilon 2}H$ being more deshielded than the $N^{\delta 1}H$. With the ability to collect higher quality spectra and to incorporate ^{15}N into subtilisin E, we have revisited the assignment problem and now report that our assignments adopted for chymotrypsin[11a] were incorrect. Most importantly, for subtilisin, we can now demonstrate that the two resonances observed *pertain to a single histidine at the active center*. We also make effective use of NOE methods to assign the $N^{\delta 1}H$ and $N^{\epsilon 2}H$ resonances in the peptideboronic acid−chymotrypsin and the peptideboronic acid−subtilisin complexes. The hypothesis of our experiments is that $N^{\epsilon 2}H$ should experience similar NOEs from both $C^{\epsilon 1}H$ and $C^{\delta 2}H$, whereas $N^{\delta 1}H$ should experience a significant NOE only to the $C^{\epsilon 1}H$. The nomenclature used is indicated below:



Experimental Section

Protein Preparation. Subtilisin E was produced by the *Bacillus subtilis* strain DB104,[13] as described previously, or DB427[15] harboring the plasmid pKWZ.[16] DB104/pKWZ was obtained from Prof. Roi Doi. The plasmid pKWZ was used to transform DB427 (obtained from Prof. S.-L. Wong) according to ref 17. Subtilisin E was purified by ion-exchange chromatography as described previously,[18] and the purity was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. For expression of ^{15}N-labeled (99% $^{15}NH_4Cl$ from Isotec, OH) subtilisin E, a minimal medium[19] was used. Each liter of medium contained 8.36 g of MOPS, 0.522 g of K_2HPO_4, 0.535 g of $^{15}NH_4Cl$, 10 g of glucose, 50 mg of tryptophan, 180 mg of histidine (for DB104 only), 125 mg of $MgSO_4$, 0.5 mM $CaCl_2$, 0.05 mM $MnCl_2$, 0.5×10^{-6} M $FeSO_4$, 3×10^{-6} mM $(NH_4)Mo_7O_{24}$, 4×10^{-4} mM H_3BO_3, 3×10^{-5} mM $CoCl_2$, 10^{-5} mM $CuSO_4$, 10^{-5} mM $ZnSO_4$, and 30 mg of kanamycin. α-Chymotrypsin, three times crystallized type II from bovine pancreas, was from Sigma (St. Louis, MO). For NMR analysis, the samples contained 1 mM enzyme inhibited with an excess amount of MeoSuc-Ala-Ala-Pro-boroPhe[20] (BoroPhe) in 20 mM sodium phosphate, 0.02% sodium azide, 90% H_2O/10%D_2O, or 99.9% D_2O at pH 6.5. The sample pH was measured at room temperature using an Orion microelectrode without correction for isotope effects.

NMR Spectroscopy. NMR spectra were recorded at 25 °C using a Varian Inova-500 spectrometer with a 5-mm triple-resonance ^{15}N/^{13}C/^{1}H probe. ^{1}H chemical shifts were referenced to DSS, and ^{15}N chemical shifts were referenced to an external sample of 2.9 M $^{15}NH_4Cl$ in 1 M HCl at 29.43 ppm.[21] Quadrature detection in all of the indirectly detected dimensions was achieved via the States-TPPI[22] method. All spectra were processed with the Varian VNMR software. An SS-shaped pulse[23] was used to suppress the water resonance in one-dimensional and one-dimensional NOE[24] experiments. The spectral width was 16 044 Hz, and 16 064 data points were collected. The recycle time was 1.5 s. Prior to Fourier transformation, a Gaussian window function was applied to the data. One-dimensional NOE experiments were performed with a 50−100-ms presaturation pulse prior to the final reading pulse. The presaturation pulse was of sufficient power to saturate the irradiated peak by about 90%. The NOE data were obtained by taking the difference between the spectrum collected with the presaturation pulse on the resonance of interest and a control spectrum collected with the presaturation pulse set off-resonance, usually 6 ppm downfield from the peak of interest. These two sets of data were interleaved to minimize subtraction error. Typically, 4096 scans were collected at each frequency offset.

The NOESY spectrum was collected using the SS read pulse. The recycle time was 1.5 s, with 512 scans collected for each t_1 increment with a spectral width of 14 057 Hz in both dimensions, 4096 data points

(14) Kawamura, F.; Doi, R. H. *J. Bacteriol.* **1984**, *160*. 442−444.
(15) Park, S. S.; Wong, S. L.; Wang F. L.; Doi, R. H. *J. Bacteriol.* **1989**, *171*, 2657−2665.
(16) Pierce, J. A.; Robertson, C. R. *Biotechnol. Prog.* **1992**, *8*, 211−218.
(17) Wang, L. F. In *Biology of Bacilli*; Doi, R. H., McGloughlin, M., Eds.; Butterworth-Heinemann: Boston, MA, 1992; pp 339−369.
(18) (a) Zhu, X.; Ohta, Y.; Jordan, F.; Inouye, M. *Nature* **1989,** *339*, 483−484. (b) Hu, Z.; Zhu, X.; Jordan, F.; Inouye, M. *Biochemistry* **1994**, *33*, 562−569. (c) Hu, Z.; Haghjoo, K.; Jordan, F. *J. Biol. Chem.* **1996,** *271*, 3375−3384.
(19) Neidhardt, F. C.; Block, P. L.; Smith, D. F. *J. Bacteriol.* **1974,** *119*, 736−747.
(20) Kettner, C. A.; Shenvi, A. B. *J. Biol. Chem.* **1984,** *259*, 15106−15112.
(21) Levy, G. C.; Lichter, R. L. *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy*; John Wiley & Sons: New York, 1979.
(22) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.
(23) Smallcombe, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 4776−4785.
(24) Wagner, G.; Wüthrich, K. *J. Magn. Reson.* **1979**, *33*, 675−680.

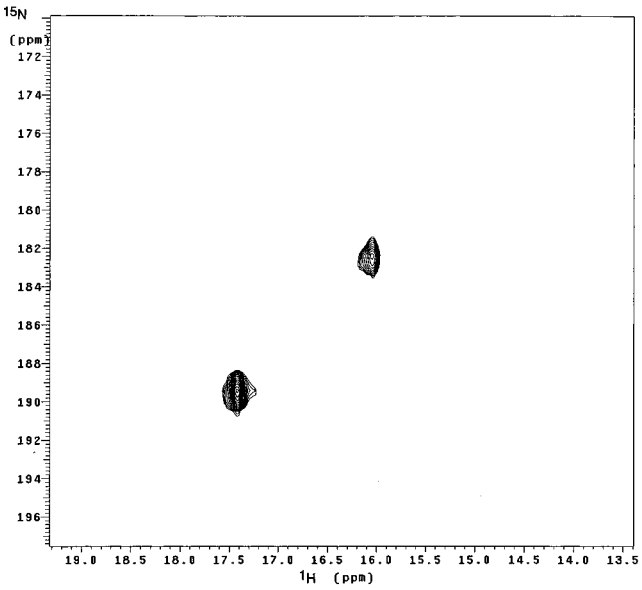


Figure 2. $^1H-^{15}N$ HMQC11 spectrum of ^{15}N-labeled subtilisin E complexed with BoroPhe. The experiment was carried out at pH 6.5 in H_2O at 25 °C.

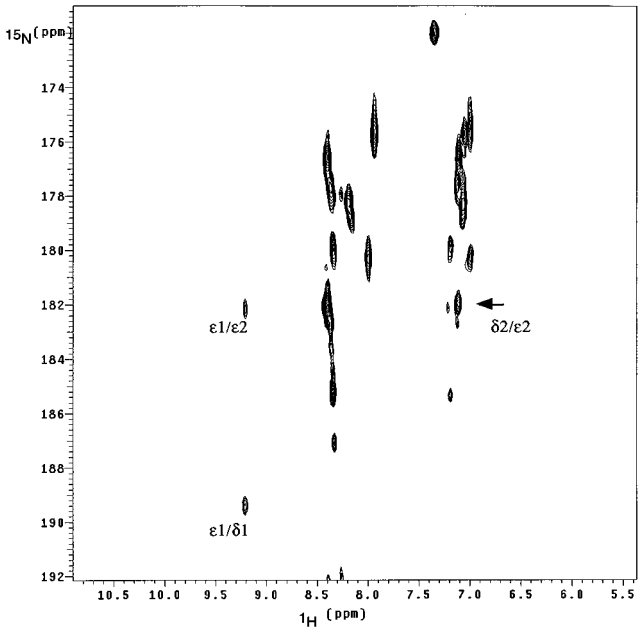


Figure 3. $^1H-^{15}N$ HMQC spectrum of ^{15}N-labeled subtilisin E complexed with BoroPhe via long-range coupling. Assignments of His64 are indicated on the spectrum.

in t_2, and 128 complex data points in t_1. The mixing time was 100 ms, and a 2-ms gradient pulse on the *z* axis was used at the end of the mixing time to remove transverse water magnetization.

In the $^1H-^{15}N$ HMQC11[24,25] experiment, proton pulses were substituted with a 11 spin−echo pulse[26] to suppress the water resonance. The spectral width was 16 044 Hz for ^1H and 7000 Hz for ^{15}N, and the carrier frequency was set at the water resonance in the ^1H and at 3776 Hz in the ^{15}N dimension. A total of 2048 complex t_2 points and 128 t_1 increments were acquired, and 256 scans were collected per t_1 increment. Broad-band GARP decoupling was applied to ^{15}N during acquisition. Prior to Fourier transformation in each dimension, the data were multiplied by a phase-shifted sine-bell window function.

The $^1H-^{15}N$ HMQC spectrum of uniformly ^{15}N-labeled protein was acquired with a delay equal to 1/(2*J*) of 22.2 ms to detect two-bond $^1H-^{15}N$ interactions while minimizing the signal from directly bonded $^1H-^{15}N$ pairs. The spectral width was 16 044 Hz for ^1H and 4560 Hz for ^{15}N. The carrier frequency was set at the water resonance for ^1H and 5120 Hz for ^{15}N. The same number of data points were acquired in both dimensions as in the HMQC11 experiment and 512 scans were collected per t_1 increment. Broad-band GARP decoupling was applied to ^{15}N during acquisition. Before Fourier transformation in each dimension, the data were multiplied by a phase-shifted sine-bell window function.

(25) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson*. **1993**, *55*, 301−315.

(26) Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc*. **1986**, *108*, 4285−4294.

Table 1. Chemical Shift Assignments (ppm) for the Imidazole of Histidine 64 of Subtilisin E or Histidine 57 of Chymotrypsin Inhibited with BoroPhe at 25 °C and pH 6.5

	$N^{\delta1}$	$N^{\delta1}H$	$N^{\epsilon2}$	$N^{\epsilon2}H$	$C^{\delta2}H$	$C^{\epsilon1}H$
subtilisin E	189.4	17.38	182.4	16.01	9.20	7.09
chymotrypsin	NA[a]	16.88	NA	16.12	9.24	7.17

[a] NA, not available.

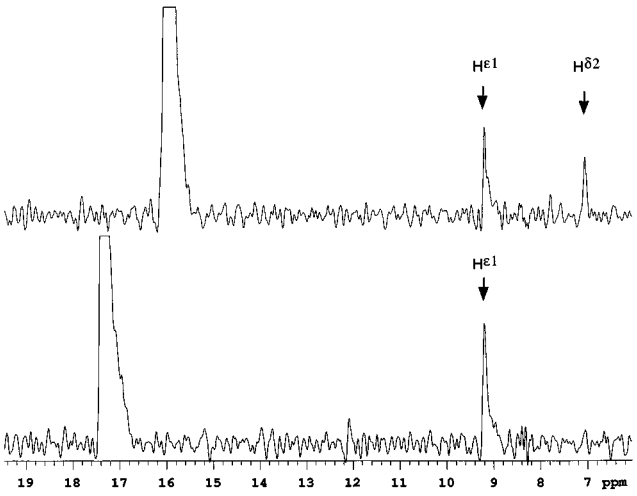


Figure 4. One-dimensional NOE spectrum of subtilisin E complexed with BoroPhe at 25 °C at pH 6.5. The largest (truncated) peak in each trace is the one irradiated. Arrows indicate the major NOE effects.

Results and Discussion

In previous work,[11] we noted that, for the complexes formed between Suc-Ala-Ala-Pro-PheB$(OH)_2$ (BoroPhe) or Suc-Ala-Ala-Pro-ValB$(OH)_2$ and chymotrypsin, between pH 5 and 9, two pH-independent proton resonances can be observed in the 15−18 ppm chemical shift region, in contrast to the single titratable resonance observed in the uncomplexed enzyme.[11a] In this study, we carried out a rigorous assignment of these resonances on the basis of both molecular genetics methods and current NMR methodologies.

In our work on the folding of subtilisin E with the assistance of its pro-sequence (such as those in refs 18 and others listed therein), we used a *B. subtilis* DB104 cell line, a highly protease-deficient histidine, and tryptophan auxotroph requiring supplementation of the minimal medium with histidine and tryptophan. When grown on [^{15}N]ammonium salts, these cells would produce no ^{15}N labeling of histidines in subtilisin E, unless ^{15}N-labeled histidines were used. To incorporate ^{15}N into the histidines of subtilisin E, we transferred the pKWZ encoding the subtilisin E gene into *B. subtilis* strain DB427 (this is a tryptophan auxotroph). The subtilisin E expressed from either strain was purified using the same procedure,[16] and the protein band found on SDS-PAGE (data not shown) was at the same position for both.

The high-frequency region of the proton NMR spectra of α-chymotrypsin (unlabeled, Figure 1A) and ^{15}N-labeled sub-

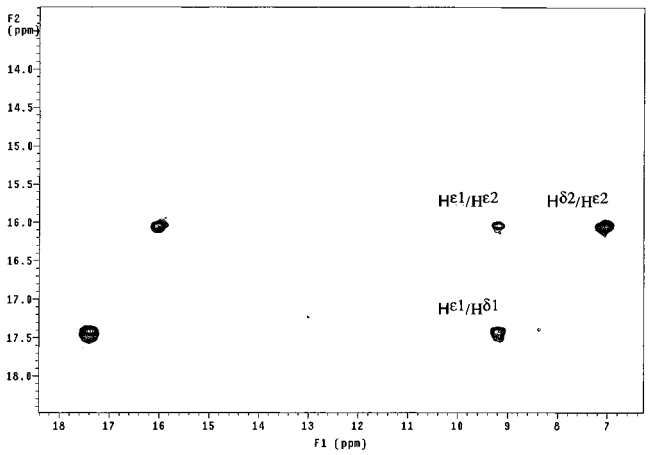


Figure 5. 2D NOESY spectrum of subtilisin E complexed with BoroPhe at 25 °C and pH 6.5. The assignments of the cross-peaks are shown.

tilisin E produced using the DB427 cell line [without ^{15}N decoupling (Figure 1B) and with ^{15}N decoupling (Figure 1C)] complexed to BoroPhe show two resonances, as reported earlier.[11] In the ^{15}N-labeled subtilisin, the two resonances appear as doublets with a *J* coupling constant of 90 Hz. The doublets collapse into singlets when ^{15}N broad-band decoupling was applied during acquisition, and the singlets have the same frequencies as those in the unlabeled protein. However, the two high-frequency resonances remain singlets (data not shown) in an undecoupled spectrum of ^{15}N-labeled subtilisin E, when produced using the DB104 cell line (histidine auxotroph) with $^{15}NH_4Cl$ as the sole nitrogen source for the minimal medium. Proton NMR spectra of subtilisin E expressed from either cell line with $^{15}NH_4Cl$ as the sole ^{15}N source with the same minimal medium showed the same resonances in the aromatic/amide chemical shift range in HSQC spectra (not shown). *The results provide strong molecular genetic evidence that the resonances in the 16−18 ppm chemical shift range pertain to histidine nitrogen-bound protons*. Since the resonances are only present at these chemical shifts in the enzyme−peptideboronic acid complexes,[11] one can further conclude that this histidine is at the active center of the enzyme.

The nitrogen-bound protons of the active center histidine of subtilisin E exchange rapidly with the aqueous solvent and cannot be observed in D_2O. In H_2O, such signals are strongly attenuated even by gradient water suppression and are only observed by selective excitation methods, such as the 11 echo and the SS pulse. Therefore, $^1H-^{15}N$ HMQC11 was used to obtain $^1H-^{15}N$ chemical shift correlation of the active center histidine of subtilisin E inhibited by BoroPhe (Figure 2). The nitrogen chemical shift of both resonances is about 180 ppm, suggesting that they pertain to protonated nitrogens of an imidazolium cation.[6b,28a,b]

The assignment of the resonances to protons on the histidine imidazole ring is based on the peak pattern in the HMQC spectrum acquired in H_2O with a delay of 22.2 ms. The ring nitrogens $N^{\delta 1}$ and $N^{\epsilon 2}$ are linked to $C^{\epsilon 1}H$ and $C^{\delta 2}H$ by weak two-bond couplings ($^2J_{N\delta 1-C\epsilon 1H}$, $^2J_{N\epsilon 2-C\epsilon 1H}$, $^2J_{N\epsilon 2-C\delta 2H}$) and give a triangular pattern. Usually, the three-bond remote coupling between $N^{\delta 1}H$ and $C^{\delta 2}H$ is weaker than the two-bond couplings and thus can be distinguished. His64 of subtilisin E inhibited with BoroPhe shows a triangular pattern of cross-peaks (Figure 3). The resonance at 183 ppm is assigned to $N^{\epsilon 2}$, and that at 189 ppm to $N^{\delta 1}$ of His64. Therefore, the resonance at 16 ppm is assigned to $N^{\epsilon 2}H$ and that at 17.4 ppm to $N^{\delta 1}H$ in the $^1H-^{15}N$ HMQC11 and in the 1D spectrum. The assignments and chemical shifts are summarized in Table 1.

This assignment is further supported by one-dimensional NOE spectra of the subtilisin E-BoroPhe complex (Figure 4). Since each NH proton in the imidazole ring of His is adjacent to one or two ring CH protons, the resonances can be assigned by NOE connectivity: the one at 17.4 ppm gives rise to one NOE peak and the one at 16 ppm to two peaks. This experiment confirms their assignments to $N^{\delta 1}H$ and $N^{\epsilon 2}H$, respectively. Two-dimensional NOESY experiments (Figure 5) lead to the same conclusions but require considerably longer acquisition time. These experiments also confirm that *all of the observed resonances pertain to the same histidine*, by no means a

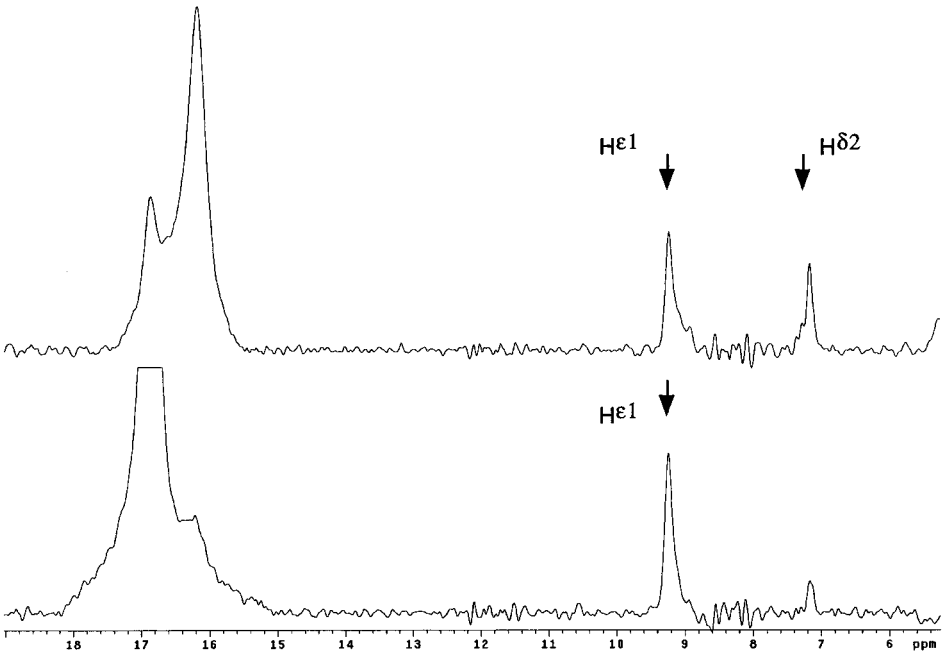


Figure 6. One-dimensional NOE spectrum of α-chymotrypsin complexed with BoroPhe at 25 °C and pH 6.5. The largest (truncated) peak in each trace is the one irradiated. Arrows indicate the major NOE effects.

foregone conclusion from the 1D spectra alone (there are six histidines in subtilisin E).

The one-dimensional NOE spectra of the chymotrypsin−BoroPhe complex (Figure 6) show very similar NOE connectivities to those in the 1D NOE spectrum of the complex with subtilisin E (compare Figures 4 and 6) and allow us to assign the 16.88 ppm resonance to $N^{\delta 1}H$ and the one at 16.12 ppm to $N^{\epsilon 2}H$. This latter assignment reverses the assignments adopted in our previous communication for chymotrypsin[11a] and points out the danger in transferring assignments among enzymes even with highly conserved active centers. Although our general conclusions in that paper were correct, the discussions on differential exchange properties of the two resonances need to be reexamined in light of these revised assignments.

A further cautionary note is in order concerning the chemical shift of the active center $C^{\epsilon 1}H$ resonances. The chemical shift of this resonance is usually quoted between 7.6 and 8.8 ppm.[29]

Repeated efforts, using spin-lock[30] and Carr−Purcell−Meiboom−Gill T_2 methods, failed to detect the 9.2 ppm resonance in these complexes. Our best explanation is that the relaxation time of such resonances in the complexes is so different from that of the other histidines as to make detection by conventional methods (those that allow us to detect the narrower $C^{\epsilon 1}H$ resonances in the presence of the broader slowly exchanging backbone NHs) fruitless. On the other hand, it is also noteworthy that the $C^{\epsilon 1}H$ resonances are quite narrow for proteins of such molecular mass (24.5 kDa for chymotrypsin and 27 kDa for subtilisin). The results also demonstrate that (1) the enzymes are still fully active—a native tertiary structure is required to observe the two hydrogens on the active center histidine and (2) the $C^{\epsilon 1}H$ resonances are indeed narrow, as claimed by us[31] and others[32] in some previous publications on subtilisins, but still questioned by some,[33] in part on theoretical grounds. Most importantly, the results show that assignments of these protons at very high frequencies can indeed be made in the absence of ^{15}N labeling with current technology.

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(27) Sklenar, V.; Bax, A. *J. Magn. Reson*. **1987**, *74*, 469−479.
(28) (a) Blomber, F.; Maurer, W.; Ruterjans, H. *J. Am. Chem. Soc*. **1977**, *99*, 8149−8159. (b) Pelton, J. G.; Torchia, D. A.; Meadow, N. D.; Roseman, S. *Protein Sci.* **1993**, *2*, 543−558.
(29) Wütrich, K. *NMR of Proteins and Nucleic Acids*; Wiley-Interscience: New York, 1986; p 17.
(30) Zhou, M. M.; Davis, J. P.; Van Etten, R. L. *Biochemistry* **1993**, *32*, 8749−8486.
(31) Jordan, F.; Polgar, L.; Tous, G. *Biochemistry* **1985**, *24*, 7711−7717.
(32) Bycroft, M.; Fersht, A. R. *Biochemistry* **1988**, *27*, 7390−7394.
(33) Consonni, R.; Molinari, H.; Greco, F.; Zannoni, G.; Zetta, L.; Carrea, G.; Riva, S. *Biochim. Biophys. Acta* **1992,** *1119*, 39−44.