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Observation of Tightly Bound ^{11}B Nuclear Magnetic Resonance Signals on Serine Proteases. Direct Solution Evidence for Tetrahedral Geometry around the Boron in the Putative Transition-State Analogues[1,2]

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Abstract: ^{11}B NMR experiments are reported for seven boronic acid inhibitors, including a very potent slow-binding peptide-based one, in the presence of excess chymotrypsin. Under these conditions, for several inhibitors slow-exchange is evident between chymotrypsin-bound and free inhibitor from the observation of separate signals only on addition of the enzyme. This new condition enables assignment of the bound boron to tetrahedral symmetry based solely on its chemical shift. Surprisingly, one of the inhibitors shows two enzyme-bound tetrahedral boron signals, only one of which is removed by acylation of the active center Ser195 with phenylmethanesulfonyl fluoride. To demonstrate the requirement for Ser for binding of the inhibitors, anhydroSer195 chymotrypsin was synthesized and found not to show any bound boron signals under any conditions tried so far. In addition to the tetrahedral site in the slow-exchange regime, there was in a few cases also demonstrated the existence of a boron resonance in fast-exchange between the free and enzyme-bound forms, in accord with a previous report from this laboratory (Adebodun, F.; Jordan, F. *J. Am. Chem. Soc.* **1988**, *110*, 309–310). These new observations are applicable to inhibitors with *any* binding affinity, so long that the boron is in slow-exchange between the free and bound forms. The study is therefore complementary to the one reported earlier, in which the boron nucleus was found in rapid exchange between the free and bound forms, necessitating extraction of the bound chemical shift and relaxation properties (to enable calculation of the quadrupolar constant and thence the hybridization) from the weighted average values.

Introduction

Boric acid and its analogues have been long recognized as inhibitors of serine proteases.[3] It was suggested on kinetic grounds[4] and also demonstrated by X-ray structural analysis[5] as well as by Raman spectroscopy[6] that phenethylboronic acid and phenylboronic acid are covalently bound in a tetrahedral geometry to the active center Ser of serine proteases. More recently, peptide boronic acids have been synthesized and their interactions with a number of proteases were evaluated.[7] In a previous study it was reported from this laboratory that under conditions of [boronic acid] ≫ [serine protease] near room temperature (22 °C), the

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(2) Presented in part: (a) the NMR Topical Group Poster Session of the North Jersey Section of the ACS at Exxon R & E, Clinton, NJ, May 1990. (b) Poster P6-20 at the XIV International Conference on Magnetic Resonance in Biological Systems, Warwick, UK, Sept. 9–14, 1990.
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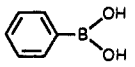
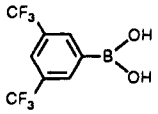
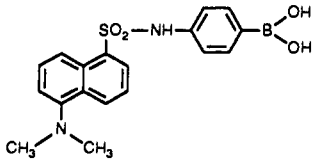
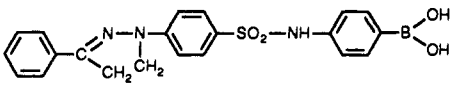
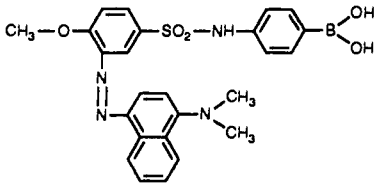
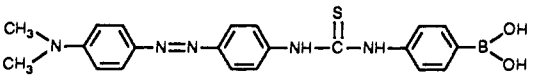
Table I. Structure of Inhibitors Used

name of inhibitors	structure of inhibitors	pK_a of inhibitors[a]
phenylboric acid, **1**		8.85
3,5-bis(trifluoromethyl)phenylboronic acid, **2**		7.19
FluoroBoro I, **3**		10.41
FluoroBoro II, **4**		9.62
ChromoBoro I, **5**		9.85
ChromoBoro II, **6**		9.68
MeOSucAlaAlaPro-boroPheOH, **7**		

[a] The pH at which there is an equimolar concentration of trigonal and tetrahedral species of the unbound inhibitor, i.e., the midpoint of transition between the two.

boron nucleus of **1** behaves as if it were in fast-exchange between the free and the enzyme-bound forms.[8] This behavior enabled estimation of the chemical shift as well as of the quadrupolar coupling constant for the enzyme-bound boron, both parameters being consistent with tetrahedral hybridization[9] around the boron atom in the active center.

Continuing our efforts to further elucidate the properties of the boron binding mechanism in the proteases, we here report conditions, such that [boronic acid] ≤ [serine protease], *and* for stronger inhibitors, under which we can observe fully bound ^{11}B NMR signals directly. For a variety of boronic acid inhibitors, including a very tightly bound peptide boronic acid, and for serine proteases from both mammalian (chymotrypsin) and bacterial (subtilisin) origin, these signals *always reflect a tetrahedral environment*. We developed this second approach inspired by an article that reported protein-bound ^{51}V (I = 7/2) signals and outlined the conditions that favor narrowing of protein-bound resonances corresponding to quadrupolar nuclei with half-integer spins.[10]

Experimental Methods

^{11}B NMR was performed at 64.21 MHz on an IBM-Bruker WP-200SY spectrometer equipped with a dedicated borosilicate glass-free probe tuned to this frequency and using 10-mm o.d. quartz tubes (Wilmad, Buena, NJ). Chemical shifts are quoted from external trimethyl borate dissolved in $CDCl_3$. All samples were made up in 2 mL total volume of 0.075 M phosphate, with 20% D_2O for a lock signal. Typical NMR experiments ran for between 20 000 and 1 million transients so as to provide satisfactory signal-to-noise ratios, with a 90° (or smaller) pulse, no recycle delay, and an ca. 460-ppm spectral window. The resonances shown in the figures represent all those observed. Concentrations of inhibitors and enzymes are in the legends.

Inhibitors used were selected to a large extent according to their commercial availability and are shown in Table I. They were obtained from the following sources: phenylboronic acid (**1**) from Aldrich; 3,5-bis(trifluoromethyl)phenylboronic acid (**2**) from Lancaster Synthesis Ltd.; FluoroBora I; 3-(dansylamido)phenylboronic acid (**3**), FluoroBora II; 3-(darpsylamido)phenylboronic acid (**4**), ChromoBora I; 3-[(((((dimethylamino)naphthyl)azo)methoxy)phenyl)sulfamido]phenylboronic acid (**5**), ChromoBora II; 3-[(((((dimethylamino)phenyl)azo)phenyl)thio)ureido]phenylboronic acid (**6**) from Polysciences, Warrington, PA; and MeOSucAlaAlaPro-boroPheOH (**7**) from DuPont Merck Pharmaceuticals. The latter differs from the tetrapeptide MeOSucAlaAlaPro-PheOH by having a $-B(OH)_2$ in place of $-CO_2H$ at its carboxyl terminus.

α-Chymotrypsin, CHT, three times crystallized type II from bovine pancreas was from Sigma, St. Louis, MO, as was subtilisin Carlsberg. The enzymes were assayed using *N*-suc-Phe-*p*-nitroanilide or *N*-acetyl-L-Tyr-ethyl ester as chromophoric substrates. Anhydrochymotrypsin was synthesized according to literature procedures[11] by first acylating the

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(5) (a) Matthews, D. A.; Alden, R. A.; Birktoft, J. J.; Freer, S. T.; Kraut, J. *J. Biol. Chem.* **1975**, *250*, 7120–7126. (b) Tulinsky, A.; Blevins, R. A. *J. Biol. Chem.* **1987**, *262*, 7737–7743.

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(9) (a) Excluding halogen containing compounds. (b) Kidd, G. R. In *NMR of Newly Accessible Nuclei*; Laszlo, P., Ed.; Academic Press: New York, 1983; Vol. 2, pp 49–77.

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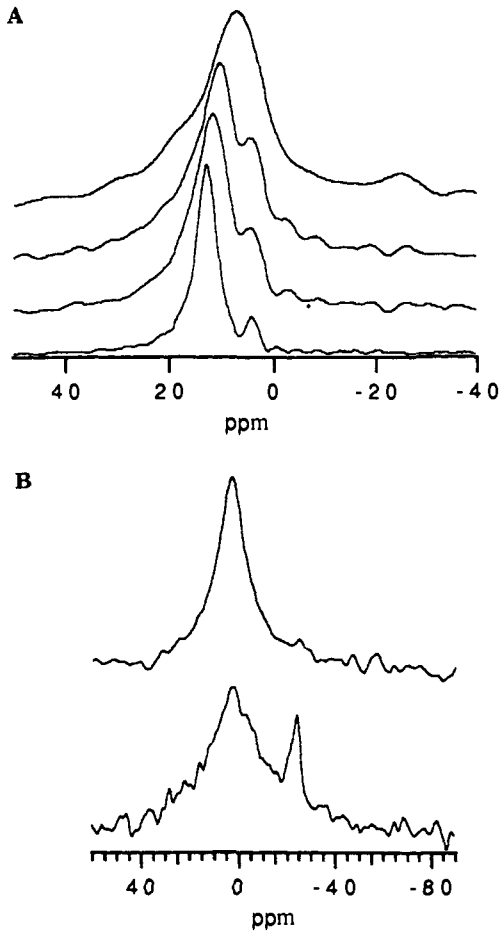


Figure 1. (A) ^{11}B NMR spectra at pH 7.0 of 1.0 mM of **1**, in the presence of (from bottom to top) 0, 0.23, 0.45, and 0.83 mM CHT at 20 °C. The small peak at 3 ppm is free boric acid contamination. (B) ^{11}B NMR spectra at pH 7.0 of 1.0 mM of **1** in the presence of 2.0 mM CHT: top at 20 °C with resonance in fast-exchange at 3.6 ppm, bottom at 5 °C with tetrahedral slow-exchange resonance at −24. ppm, fast-exchange one at 3.0 ppm.

active center with phenylmethanesulfonyl fluoride and then reacting with hydroxide to produce the anhydroSer195 (dehydrated) derivative. This anhydroSer195 chymotrypsin derivative was purified by affinity chromatography on a bovine pancreatic trypsin inhibitor matrix. According to this criterion, the anhydrochymotrypsin retained structure, although it was devoid of catalytic activity.

Results and Discussion

As will be apparent, there is strong evidence that for several inhibitors studied the binding is both temperature- and pH-dependent; therefore, data will be presented for at least two temperatures. As a point of departure for the ensuing discussion, the chemical shifts are quoted (ppm, against the external standard used throughout, trimethyl borate in $CDCl_3$) for the trigonal and tetrahedral forms, respectively, of the unbound species: boric acid, 3,8 and −15 ppm; compound **1**, 14,1 and −12.4; compound **2**, 13.4 and −13.2 ppm; compound **3**, 4 and −12.0; compound **4**, 3,9 and −13,4; compound **5**, 4,0 and −13,5; compound **6**, 3.8 and −13.3; and compound **7**, 3.2 and −13.1 ppm. The p*K*s for trigonal-to-tetrahedral interconversion of the unbound inhibitors are listed in Table I. For the most part, measurements were made at pH 7, at which all inhibitors, except **2**, overwhelmingly favor their trigonal structures. Specific chemical shifts are difficult to glean from the spectra because of the line widths and therefore are listed in the figure legends. The chemical shifts are also subject to significant error, ±0.5–1.0 ppm for the tetrahedral "bound" resonances and even larger for the "unbound" or exchanging resonance.

Figure 1A shows "titration" of ca. 1 mM of **1** with CHT at pH 7.0 and 20 °C. The result is similar to that previously reported

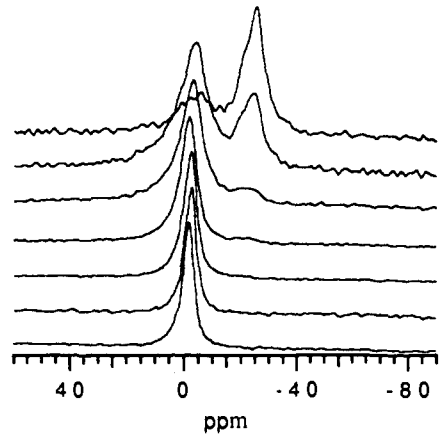


Figure 2. ^{11}B NMR spectra resulting from the addition of (from bottom to top) 0.0, 0.02, 0.2, 0.4, 0.8, 1.6, and 3.2 mM of CHT to a solution of **2** at 4.0 mM at pH 7, 20 °C.

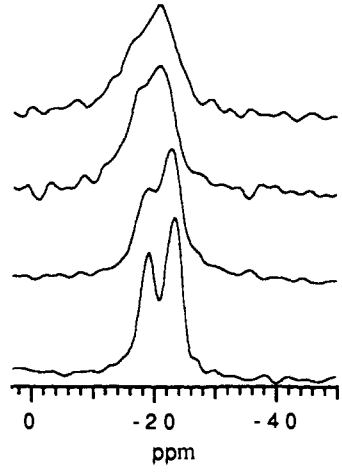


Figure 3. ^{11}B NMR spectra at pH 7.0 of 1 mM of **2**, 2.0 mM CHT, at (bottom to top) 5, 10, 15, and 20 °C; slow-exchange bound resonances at −18.4 and −22.5 ppm.

(except in the previous report there was 3.0 mM of **1** and no more than 0.4 mM CHT), showing a small upfield chemical shift and broadening on addition of CHT. The small resonance near 4 ppm corresponds to boric acid contaminant. At this temperature the boron is in fast-exchange between free and enzyme-bound forms, Figure 1B shows the spectrum of 1 mM of **1** in the presence of excess CHT (2 mM) at pH 7,0 once the temperature is lowered from 20 to 5 °C. At the lower temperature a small peak with chemical shift characteristic of a tetrahedral environment becomes apparent, that coexists with a rather broad resonance downfield from the bound one, whose chemical shift is significantly upfield from that of the unbound inhibitor in the absence of enzyme at this pH. Therefore, we assign the broad resonance to inhibitor in fast-exchange between some bound form(s) and the unbound form, analogously to the previous report.[8] The data suggest that there are two different binding mechanisms (perhaps also binding sites) for binding **1** to CHT: one site in the slow-exchange regime, visible at lower temperature for a concentration ratio of [**1**]:[CHT] of 1 to 2 mM, the other in the fast-exchange regime.

Figure 2 shows the detailed behavior of 4 mM of **2** on incremental addition of CHT at 20 °C. Two features are apparent. First, the resonance in the "trigonal" region exhibits an upfield chemical shift on incremental addition of enzyme, indicative of a boron in fast-exchange, similar to that discussed for **1** above. In addition, however, there is also evidence for a resonance that is in slow-exchange, once the concentration ratio of CHT to **2** approaches unity. Figure 3 demonstrates the temperature dependence of the spectrum of a mixture of 2 mM of CHT and 1 mM of **2** at pH 7. This compound is reported to be a much stronger inhibitor of subtilisin than is **1** (K_d = 1 μM),[13] Ap-

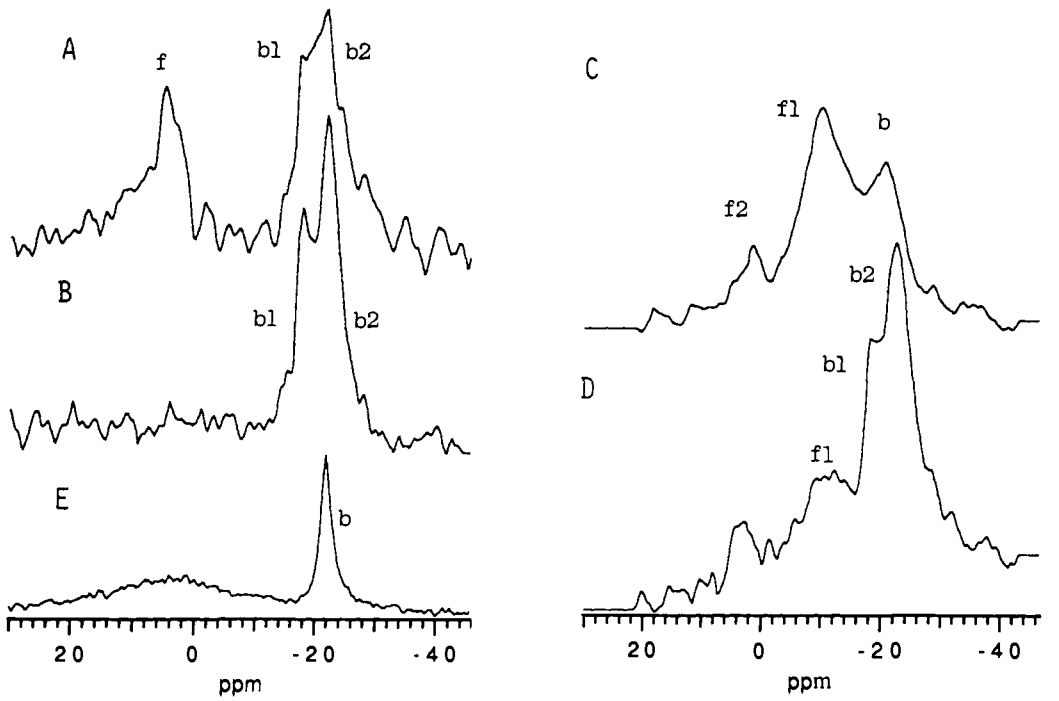


Figure 4. Boron resonances for **2** bound to CHT under different conditions. Spectrum A: 2 mM of **2**, 2 mM CHT, pH 6.06, 20 °C; bound resonances at ca. −18.1 (b1) and −22.8 ppm (b2), unbound or fast-exchange at 3.8 ppm (f). Spectrum B: 2.0 mM of **2**, 2.0 mM CHT, pH 6.5, 0 °C; bound resonances at −18.6 (b1) and −22.9 ppm (b2). Spectrum C: 2.0 mM of **2**, 2.0 mM CHT, pH 8.16, 20 °C; bound resonance at ca. −21.7 ppm (b), fast exchange one at −11.9 (**2**, f1) and 0.2 (boric acid, f2) ppm. Spectrum D: 2.0 mM of **2**, 2.0 mM CHT, pH 7.85, 0 °C; bound resonances at ca. −18.3 (b1) and −22.8 ppm (b2), unbound ones at −10.5 (**2**, f1) and 2.9 (boric acid) ppm. Spectrum E: 0.6 mM of **2**, 2.0 mM CHT, 0.075 M phosphate, pH 7.0, 5 °C; incubated with 3.6 mM phenylmethanesulfonyl fluoride, bound resonance at ca. −21.8 ppm (b).

parently, it is also a much more potent inhibitor of CHT as well, since at lower temperature there is evidence only for two bound species, both tetrahedral. We can be certain that both of the resonances observed pertain to enzyme-bound species, since their chemical shifts are well upfield from that observed for even fully tetrahedral **2** in the absence of enzyme. From these lower temperature spectra we thus conclude that there are *two tetrahedral* enzyme-bound forms for this compound in the slow-exchange regime. When the experiment is repeated with phenylmethanesulfonyl (PMS) fluoride treated CHT, the resonance at lower field (−18.5 ppm) disappears (Figure 4E). Since PMS-CHT has an acylated Ser195,[14] we assign the −18.5 ppm resonance to the boron bound to the active center Ser195. Figure 4 demonstrates that the appearance of such spectra strongly depends on pH, in addition to the temperature-dependence shown in Figure 3. The slow exchange nature of the phenomenon studied is clear in Figure 4A, that has both "free" and bound inhibitor in the same spectrum. Presumably, the binding is stronger at lower temperature, hence at 0 °C only the two resonances corresponding to bound inhibitor are observed. When the pH is raised to above the His57 pK of ca. 6.8, it is more difficult to observe the bound signal at 20 °C, while two resonances are still clearly visible at the higher pH once the temperature is lowered (Figure 4C,D). While the resonance at −18.5 ppm corresponds to Ser195-bound boron, that at higher field may represent boron bound to His (as suggested by others elsewhere)[7d,f,g] or, perhaps, the tetrahedral boronate adduct formed with hydroxide ion and stabilized in the active center by electrostatic interaction with the $HisH^+$ (note the very low pK for trigonal-to-tetrahedral transition of this inhibitor alone). Since PMS at Ser195 should introduce a significant steric hindrance to binding at His57, we currently favor the second explanation.

While this manuscript was under review, there appeared a note reporting ^{11}B NMR results basically parallel to those here discussed for compound **2** only, in the presence of CHT.[15] Curiously, those authors failed to notice the fact that there are two resonances in the slow-exchange regime with chemical shifts corresponding to tetrahedrally bound boronates. There may be several reasons for this: they used a 400-MHz instrument at 23 °C and only in a very limited pH range; we used a 200-MHz one at a variety of temperatures and pH values. Nevertheless, in Figure 1A of that report, there is a distinct shoulder on the "bound" resonance, apparently disregarded by those authors, exactly as we observed at higher temperature.

Figure 5 shows the temperature-dependent behavior of ^{11}B spectra for compounds **3** and **4** (at 1 mM) in the presence of 2 mM CHT. Both of these inhibitors give rise to bound, tetrahedral resonances that are in slow-exchange, and the fraction of bound inhibitor increases with lowered temperatures.

Figure 6 shows that **5** binds so weakly that there is only a small slowly exchanging resonance even at the lower temperature, while the related compound **6** exhibits a resonance in slow-exchange at both temperatures and shows enhanced binding at lower temperature.

The behavior of the "slow-binding" (this is a kinetic term characterizing a type of tight inhibitor, for which inhibition sets in with time, and is not to be confused with a resonance in the slow-exchange limit) inhibitor **7** was also examined (Figure 7).[7a] In the presence of CHT, only a resonance in the tetrahedral chemical shift range is visible. Furthermore, addition of PMSF to a solution consisting of CHT and **7**, clearly displaced some of the bound **7** from the active center (Figure 7B, recorded immediately on adding PMSF), affirming its requirement for active Ser195.

To assess the generality of the findings, compound **3** at 1 mM concentration was also studied at pH 7.0 and 20 °C in the presence of excess subtilisin Carlsberg (1.8 mM) and found to yield a single bound chemical shift of −20.8 ppm, clearly tetrahedral (Figure 8).

To probe the requirement for Ser195 in binding (i.e., whether any of the inhibitors tested could still be bound to the active center

(12) In ref 8b compound **2** was also used but did not allow quantitative interpretation because the binding curves could not be fitted to a single site.

(13) Philipp, M.; Bender, M. L. *Mol. Cell. Biochem.* **1983**, *51*, 5–32.
(14) Gold, A. M.; Fahrney, D. *Biochemistry* **1964**, *3*, 783–791.
(15) Baldwin, J. E.; Claridge, T. D. W.; Derome, A. E.; Schofield, C. J.; Smith, B. D. *Biochem. Med. Lett.* **1991**, *1*, 9–12.

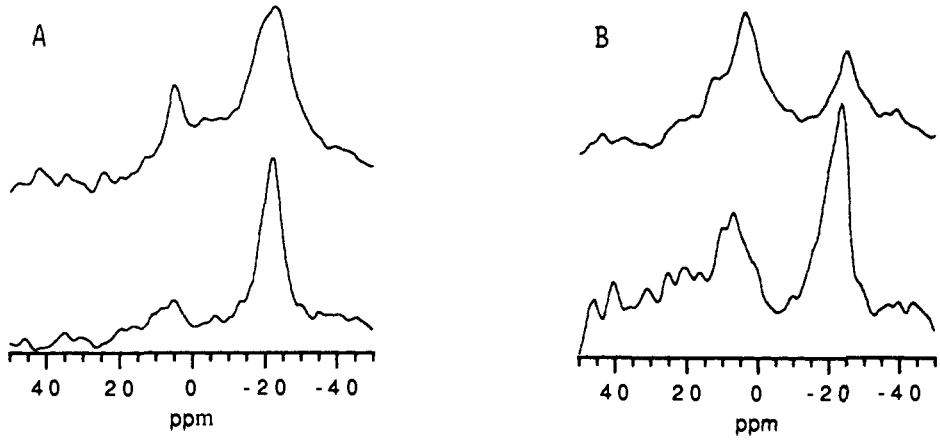


Figure 5. (A) ^{11}B NMR spectra of 1.0 mM of **3**, 2.0 mM CHT, pH 7.0: top at 20 °C, bound resonance at −22.1 ppm, fast-exchange (or unbound) resonance at 4.5 ppm; bottom at 5 °C, bound resonance at −23.1 ppm, fast-exchange (or unbound) resonance at 2.1 ppm. (B) ^{11}B NMR spectra of 1.0 mM of **4**, 2.0 mM CHT, pH 7.0: top at 20 °C, bound resonance at −25.2 ppm, fast-exchange (or unbound) resonance at 3.4 ppm; bottom at 5 °C, bound resonance at −23.4 ppm, fast-exchange (or unbound) resonance at 5.2 ppm.

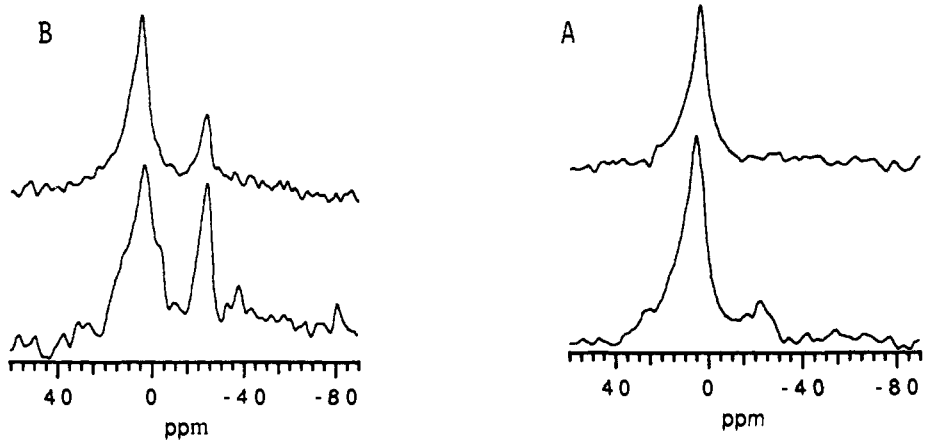


Figure 6. (A) ^{11}B NMR spectra of 1.0 mM of **5**, 2.0 mM CHT, pH 7.0: top at 20 °C, fast-exchange (or unbound) resonance at 3.8 ppm; bottom at 5 °C, bound resonance at −22.1 ppm, fast-exchange (or unbound) resonance at 3.9 ppm. (B) ^{11}B NMR spectra of 1.0 mM of **6**, 2.0 mM CHT, pH 7.0: top at 20 °C, bound resonance at −23.4 ppm, fast-exchange (or unbound) resonance at 3.9 ppm; bottom at 5 °C, bound resonance at −22.9 ppm, fast-exchange (or unbound) resonance at 3.4 ppm.

His in the absence of Ser195), anhydroSer195 chymotrypsin was synthesized[11] and showed *no tetrahedrally bound boron signal for 2 or 3 under a variety of conditions*.[16] Assuming that the active center environment in the anhydroenzyme is still intact (our only criterion for this is its ability to be bound to a protein protease inhibitor matrix), this suggests that the SerOH contributes very significantly to the binding process, since under the conditions of the experiment we observed no bound signal even at 5 °C. This experiment demonstrates that while the boron may be bound to Ser195 or to His57 and Ser195 simultaneously, the presence of intact Ser195 is *absolutely required* either for binding or to maintain an intact active center environment. Tsai and Bender had reported that anhydroelastase, in which the active center Ser was subjected to dehydration, as in this study, bound strong inhibitors with K_is well above 10 mM,[17] supporting our contention. It is also relevant to mention that mutation of the active center Ser221 to Ala in subtilisin led to a dimenution of k_{cat}/K_M by a factor of a million.[18]

A comment concerning the line width of the bound signals in the slow-exchange regime is also in order. It was suggested on the basis of theoretical considerations that the line width of quadrupolar nuclei with odd-half spins should narrow[10] on (a) cooling the solution, (b) attaching the nucleus to a macromolecule, and (c) increasing the viscosity of the solution. Indeed, there is evidence in the spectra that the bound tetrahedral resonance (resonance position is pH and concentration independent) becomes

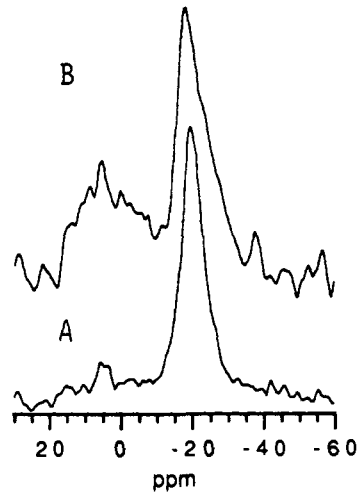


Figure 7. (A) ^{11}B NMR spectra at 5 °C of 1.6 mM of **7** in the presence of 1.4 mM CHT at pH 7.3; resonance at −20.1 ppm. (B) The spectrum of the mix in Figure 7A at 5 °C on addition of 6.8 mM phenylmethanesulfonyl fluoride, recorded immediately after mixing; resonance at −21.7 ppm.

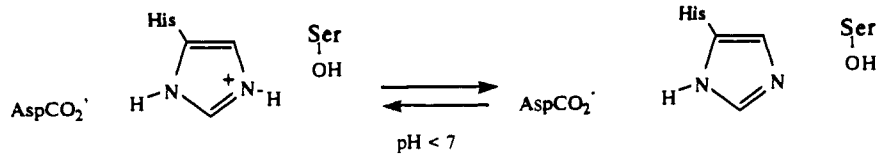
(16) The experiment used 3.2 mM anhydrochymotrypsin, 1.6 mM of **2** at pH 7.0 at 0, 20, and 30 °C as well as 0.5 mM anhydrochymotrypsin and 0.5 mM of **3** at pH 7.0 at 5 °C.

(17) Tsai, I.-H.; Bender, M. L. *Arch. Biochem. Biophys.* **1984**, *228*, 555–559.

(18) Wells, J. A.; Estell, D. E. *TIBS* **1988**, *13*, 291–297.

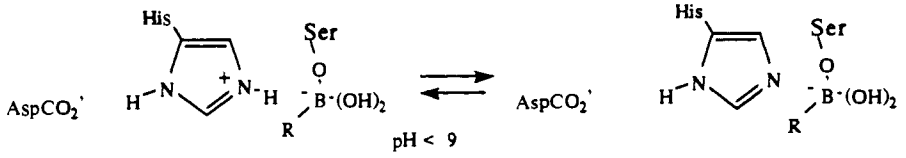
Scheme I. Possible Interactions between Serine Protease and Boronic Acids

Native Enzymes

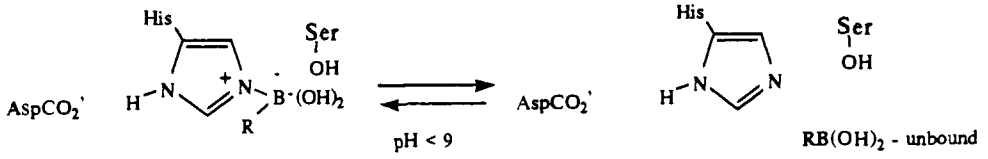


Tetrahedral Boronates

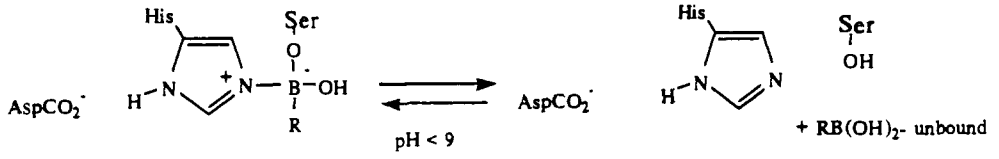
a. Bound to Ser



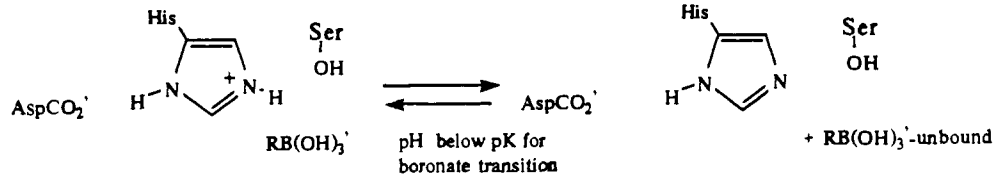
b. Bound to His



c. Bound to His and Ser concurrently



d. Non-covalent hydrated boronic acid



narrower at lower temperatures (see Figures 3, 5, and 6) as suggested for spin $^3/_2$ nuclei.[19] In addition, the boron nucleus for the same inhibitor (3) when bound to subtilisin gives rise to a narrower slow-exchange resonance than when bound to chymotrypsin (contrast Figures 5A and 8). This is unlikely to be the result of the known dimerization of chymotrypsin at the high concentrations used for the NMR experiments. In the same figures the free inhibitor gave rise to resonances with similar line widths, hence the greater line width of the slow-exchange boron resonance on CHT is specific to this enzyme.

The chemical shift of the bound tetrahedral resonance is interesting, always significantly upfield (4–9 ppm) from the resonance position of the corresponding free species in their tetrahedral forms. This finding applies to every inhibitor and to both enzymes studied. The origin of this upfield chemical shift is not clear but may, in part, be related to the second-order dynamic frequency shift experienced in the slow motion limit, reported for other quadrupolar nuclei.[20] For example, at 64 MHz for the spin $I = {}^3/_2$ the change in chemical shift $\Delta\omega_d$ is given by

$$\Delta\omega_d = 25 \times 10^{-3}\,\chi^2/\omega_o$$

where χ is the quadrupolar coupling constant, and ω_o is the spectrometer frequency.[21] The quadrupolar coupling constant has been reported to be near 0.7 MHz for tetrahedral boronates in small molecules,[22] while we estimated 0.87 MHz for boron bound to chymotrypsin[8a] and ca. 1 MHz for boron bound to subtilisin.[8b] In other words, one can readily account for as much

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(20) Neurohr, K. J.; Drakenberg, T.; Forsen, S. In *NMR of Newly Accessible Nuclei*; Laszlo, P., Ed.; Academic Press: New York, 1983; Vol. 2, pp 242–245.

(21) (a) Werbelow, L. G. *J. Chem. Phys.* **1979**, *70*, 5381–5383. (b) Werbelow, L. G. *J. Magn. Res.* **1980**, *43*, 443–448. (c) Werbelow, L. G. *J. Phys. Chem.* **1981**, *85*, 3887–3891. (d) Westlund, P.-O.; Wennerstrom, H. *J. Magn. Reson.* **1982**, *50*, 451–466.

(22) Cuthbert, J. D.; Macfarlane, W. T.; Patch, H. E. *J. Chem. Phys.* **1965**, *43*, 173–177.

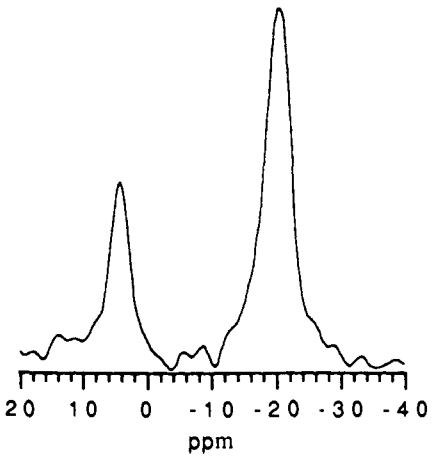

Figure 8. ^{11}B NMR spectrum of 1.0 mM of **3** and 1.8 mM of subtilisin Carlsberg at pH 7 and 20 °C; unbound resonance at 3.9 ppm, bound one at −20.1 ppm.

as 3–6 ppm of the upfield shift based on this simple estimate. Of course, in addition, the enzyme active center may have a contribution of its own, such as a solvent effect, to cause the additional shift. On the basis of the slightly different chemical shifts for different inhibitors bound to the same enzyme, the former explanation perhaps is more plausible, i.e., the second-order dynamic frequency shift may differ for different inhibitors. There is also noticed a consistently larger upfield shift (1–2 ppm) experienced by the same boronic acid inhibitor when bound to CHT, than when bound to subtilisin.

Scheme I shows possible models to account for the observations. First, based on previous proton NMR data[22] we had concluded that boronate complexes of subtilisin were stable to at least pH 9.5. The resonances observed in the slow-exchange regime undoubtedly represent tetrahedrally hybridized boron bound to Ser195 (model **a** in Scheme I), since addition of PMSF eliminated this resonance, and anhydroSer195 CHT could no longer bind even the stronger inhibitors. Compounds **1** and **2** also give rise to a boron resonance in the fast-exchange regime,[8,12] as also reported earlier. The chemical shift of the bound species extracted from the fast-exchange data was calculated to be ca. −12.9 ppm for **1**.[8a] On the basis of the chemical shift, and the suggestion that the off-rate for His-bound boron is faster than for Ser-bound boron,[4a] we tentatively suggest that the resonance in fast-exchange corresponds to model **b** in Scheme I. For compound **2** only, there was observed a second boron resonance with chemical shift characteristic of tetrahedral hybridization near −21 ppm, that was not eliminated by inactivation of the enzyme with PMSF. Because of the chemical shift and our assumption that PMS-Ser195 CHT should also sterically hinder model **b**, we suggest that this resonance may correspond to model **d**, i.e., the tetrahedral boronate sitting in the active center, stabilized by the nearby $HisH^+$. There are some reasons that make this a possible scenario: (a) the relative ease with which **2** is converted to its tetrahedral monoanionic hydrate and (b) the possibility, at least, of binding the bis(trifluoromethyl)phenyl ring in the "aromatic pocket" of CHT (the latter is by no means a requirement for this model). We had earlier demonstrated that any negative charge, covalently or noncovalently attached to the active center, will experience a favorable mutual stabilization with the $HisH^+$.[8b,23]

The results here presented will be useful in determining the binding of boron-based inhibitors, including the very potent peptide boronic acids synthesized by Kettner and co-workers,[7] to enzymes, in general, since the interpretation of the chemical shift is much easier under slow-exchange conditions. The findings complement the previous one. While our former methodology for estimating the symmetry around the enzyme-bound boron nucleus was applicable to weak inhibitors, the observations here reported are applicable to tight-binding, including "slow-binding" inhibitors. ^{11}B NMR is proving to be an excellent technique with which to probe the symmetry and binding locus of these inhibitors at the active centers of the proteases.

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