^{11}B NMR Spectroscopy of Peptide Boronic Acid Inhibitor Complexes of α-Lytic Protease. Direct Evidence for Tetrahedral Boron in both Boron–Histidine and Boron–Serine Adduct Complexes†

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ABSTRACT: We have previously shown, using ^{15}N and ^{1}H NMR spectroscopy, that MeOSuc-Ala-Ala-Pro-boroPhe and certain other boronic acid inhibitors form boron–histidine adducts with α-lytic protease instead of transition-state-like tetrahedral boron–serine adducts as is generally supposed [Bachovchin, W. W., Wong, W. Y. L., Farr-Jones, S., Shenvi, A. B., & Kettner, C. (1988) *Biochemistry* 27, 7689–7697]. An X-ray crystallographic study of the MeOSuc-Ala-Ala-Pro-boroPhe complex with α-lytic protease [Bone, R., Frank, D., Kettner, C. A., & Agard, D. A. (1989) *Biochemistry 28*, 7600–7609] has confirmed the existence of the boron–histidine bond but has concluded that the boron atom is trigonal rather than tetrahedral. Here we report a ^{11}B NMR study at 160.46 MHz of this histidine adduct complex and of two other complexes known to be serine adducts: α-lytic protease with MeOSuc-Ala-Ala-Pro-boroVal and chymotrypsin with MeOSucAla-Ala-Pro-boroPhe. The ^{11}B NMR chemical shifts demonstrate that the boron atom is tetrahedral in both the histidine and serine adduct complexes. A comparison of our results with those of a previous ^{11}B NMR study of chymotrypsin complexed to MeOSuc-Ala-Ala-Pro-boroPhe at a lower magnetic field strength (64.21 MHz) [Zhong, S., Jordan, F., Kettner, C. A., & Polgar, L. (1991) *J. Am. Chem. Soc. 113*, 9429–9435] indicates that the motional properties of the enzyme-bound ^{11}B nucleus place it outside of the extreme narrowing region and that the difference in resonance frequency between the free and the enzyme-bound tetrahedral boron can largely be attributed to a second-order dynamic frequency shift.

Peptide boronic acids are exceptionally potent inhibitors of serine proteases. K_i values of these inhibitors for their target enzymes typically range from nanomolar to picomolar. Some examples of biologically important proteases for which specific and potent boronic acid based inhibitors have been developed include α-lytic protease ($K_i = 0.35 \times 10^{-9}$ M) (Kettner et al., 1988), elastase ($K_i = 0.25 \times 10^{-9}$ M), chymotrypsin ($K_i = 0.16 \times 10^{-9}$ M), and cathepsin G ($K_i = 21 \times 10^{-9}$ M) (Kettner & Shenvi, 1984), the IgA proteases from *Hemophilus influenzae* and *Neisserria gonorrhoeae* ($K_i = 4 \times 10^{-9}$ M) (Bachovchin et al., 1990), thrombin ($K_i = 3.6 \times 10^{-12}$ M) (Kettner et al., 1990), and dipeptidyl aminopeptidase type IV (DP IV) ($K_i = 16 \times 10^{-12}$ M) (Flentke et al., 1991; Gutheil & Bachovchin, 1993). Owing to the large number of serine proteases that have been demonstrated to play crucial roles in biological systems, the high affinity and specificity achievable with boronic acid based inhibitors make them of considerable interest both as research tools and as potential therapeutic agents.

The prevailing explanation for the high affinity of these compounds for serine proteases is that the boronyl group is an exceptional good mimic of the transition state of the enzyme-catalyzed reaction and therefore binds tightly to the site on the enzyme designed to stabilize the tetrahedral transition state. However, we have recently shown that boronic acid based inhibitors do not always form the putative transition-state-like tetrahedral adduct with the active-site serine. In some complexes the boron atom of the inhibitor forms a covalent bond with the $N^{\epsilon 2}$ nitrogen of His57.[1] We first demonstrated the existence of such histidine adducts in complexes of α-lytic protease with MeOSuc-Ala-Ala-Pro-boroPhe,[2] Boc-Ala-Pro-D-boroVal, and benzeneboronic acid using ^{15}N NMR spectroscopy of ^{15}N-labeled His57 α-lytic protease (Bachovchin et al., 1988). This work also demonstrated that putative transition-state-like adducts with Ser195 were formed with Boc-Ala-Pro-boroVal, MeOSuc-Ala-Ala-Pro-boroAla, MeOSuc-Ala-Ala-Pro-boroVal, and Ac-Pro-boroVal. Comparing the structures of boronic acid inhibitors that form serine adducts with those that form histidine adducts suggested a rule governing the type of adduct obtained: Inhibitors able to form favorable contacts with the specificity subsites form serine adducts whereas those that cannot form histidine adducts. We have more recently shown that this behavior is not unique to α-lytic protease as trypsin also forms histidine adducts with nonsubstrate analog boronic acids but serine adducts with substrate analog peptide–boroArg inhibitors (Tsilikounas et al., 1992).

Although the ^{15}N and ^{1}H NMR results were unambiguous in identifying and distinguishing between histidine and serine adduct complexes, they did not provide direct information on

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[1] The chymotrypsinogen numbering system is used when specifying the residues of the catalytic triad in α-lytic protease and other enzymes of the chymotrypsin family.

[2] Abbreviations: MeOSuc, methoxysuccinyl; Boc, *tert*-butyloxycarbonyl; Ac, acetyl; FID, free induction decay; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid. The prefix boro indicates that the carboxylate of the amino acid residue is replaced by $-B(OH)_2$.

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the geometry of the boron atom. However, by showing that His57 remains protonated in the complexes at pH values as high as 10.5, which is well above its normal pK_a of 7.0 in the resting enzyme (Bachovchin & Roberts, 1978), the NMR results at least indirectly supported the existence of a tetrahedral and negatively charged boronyl group because a tetrahedral boronyl group would more readily explain the unusual stability of a protonated His57 in both types of complexes. An X-ray crystallographic study of the α-lytic protease complexed with MeOSuc-Ala-Ala-Pro-boroPhe confirmed the existence of the histidine–boron bond but concluded that the boron atom in this complex was trigonal rather than tetrahedral (Bone et al., 1989). X-ray crystallographic studies agree with NMR studies in indicating that the complexes of α-lytic protease with Boc-Ala-Pro-boroVal, MeOSuc-Ala-Ala-Pro-boroAla, and MeOSuc-Ala-Ala-Pro-boroVal are tetrahedral serine adducts (Bone et al., 1987, 1989).

To obtain direct information about the symmetry of the boron atom in both histidine and serine adduct complexes of α-lytic protease with peptide boronic acid inhibitors, we undertook a ^{11}B NMR study of these complexes. The ^{11}B NMR signal should be especially informative and unambiguous in distinguishing between trigonal and tetrahedral geometry about the boron atom (Philipp & Bender, 1971; Kidd, 1983; Baldwin et al., 1991; Zhong et al., 1991). A ^{11}B NMR study of a peptide boronic acid inhibitor–serine protease complex has been previously reported, that of chymotrypsin with MeOSuc-Ala-Ala-Pro-boroPhe (Zhong et al., 1991). This work showed the enzyme-bound boron to be tetrahedral, but it did not address the question of the boron geometry in histidine adduct complexes because on the basis of the above rule this complex is expected to be a serine adduct, and this expectation has been confirmed by ^{1}H NMR (Farr-Jones and Tsilikounas, unpublished data). ^{11}B NMR studies of several other aryl boronic acid inhibitor complexes with chymotrypsin and subtilisin also showed tetrahedral boron, but whether these complexes are histidine or serine adducts has not been established (Baldwin et al., 1991; Zhong et al., 1991).

Here we report a ^{11}B NMR study at 160.46 MHz of α-lytic protease complexed to MeOSuc-Ala-Ala-Pro-boroPhe, the histidine adduct proposed by Agard and co-workers to have a trigonal boron (Bone et al., 1989), and of α-lytic protease complexed to MeOSuc-Ala-Ala-Pro-boroVal, which NMR and crystallographic studies agree is a tetrahedral serine adduct (Bachovchin et al., 1988; Bone et al., 1989). We have also examined the complex of chymotrypsin with MeOSuc-Ala-Ala-Pro-boroPhe which has previously been studied by ^{11}B NMR but at lower magnetic field strength (Zhong et al., 1991). A comparison of our results at higher magnetic field with those of the previous study at lower magnetic field provides some interesting insights into the motional properties of the enzyme-bound boron atom and an experimental demonstration of a second-order dynamic frequency shift for a ^{11}B NMR signal, a phenomenon predicted by theory for quadrupolar nuclei and previously experimentally observed in NMR spectra of the spin $^7/_2$ vanadium nucleus (Butler & Eckert, 1989).

MATERIALS AND METHODS

α-Lytic protease (EC 3.4.21.12) was purified from *Lysobacter enzymogenes* as previously described (Bachovchin & Roberts, 1978). The enzyme was further desalted on a Sephacryl S-200 column (Pharmacia, Piscataway, NJ) equilibrated in 0.1 M KCl. The enzyme was eluted off the column and concentrated down to the volume needed for the NMR experiments using a stirred cell (Amicon, Beverly, MA). Enzyme assays were performed at 410 nm using Ac-Ala-Pro-Ala-*p*-nitroanilide (Bachem, Torrance, CA) (Bachovchin et al., 1988).

The synthesis of unpinacolated MeOSuc-Ala-Ala-Pro-L-boroPhe (-boroPhe) and MeOSuc-Ala-Ala-Pro-(D,L)boroVal (-boroVal) has been previously described (Kettner & Shenvi, 1984). –BoroVal and –boroPhe are both very potent inhibitors of α-lytic protease with K_i values of 6.4 ± 1.0 and 540 ± 63 nM, respectively, at pH 7.5 (Kettner et al., 1988).

NMR samples typically contained about 2 mM α-lytic protease or α-chymotrypsin (type II; Sigma Chemical Co., St. Louis, MO) in 1.5 mL of 0.1 M KCl. Two hundred microliters of D_2O was added to the samples to provide the field frequency lock signal. The inhibitors were directly added to the NMR samples. NMR samples of enzyme-free inhibitors were prepared by dissolving about 2 mM inhibitors in 1.5 mL of 0.1 M KCl and 200 μL of D_2O. The pH of the NMR samples was varied by adding 0.25 M NaOH or 0.25 M HCl.

^{11}B NMR spectra were recorded at room temperature on a Bruker AMX-500 spectrometer equipped with a broadband probe in which the Pyrex insert and Dewar were replaced with quartz to minimize the background boron signal. Samples were prepared in quartz NMR tubes (Wilmad Glass Co., Inc., Buena, NJ). Spectra were acquired at 160.46 MHz using 8K data points, a 90° pulse of 19 μs, a sweep width of 20 000 Hz, and a recycle delay of 0.3 s. Chemical shifts were referenced to a solution of boric acid in 90% H_2O/10% D_2O. The spectra represent between 5000 and 15 000 scans. Convolution difference (with line broadenings of 10 and 100 Hz) was applied to the FIDs to help remove the remaining background ^{11}B signal. Spectra were plotted from a Sun Sparc station computer using Felix 2.0 (Biosym Technologies, San Diego, CA). The minor peak at around –13.5 ppm which appears in certain spectra is due to an impurity in the inhibitors.

RESULTS

MeOSuc-Ala-Ala-Pro-boroVal. Figure 1 shows the ^{11}B NMR spectra of free -boroVal in solution as a function of pH. The inhibitor shows one broad signal at –16.5 ppm (Figure 1A) at high pH which disappears as the pH is lowered and is replaced by a signal at +0.1 ppm (Figure 1D–F). The chemical shifts of the high- and low-pH forms are quite typical of tetrahedral and trigonal forms, respectively, of the boronyl group (Zhong et al., 1991). The pK_a of about 9, estimated from the spectra of Figure 1, is also typical for the transition between the tetrahedral and trigonal forms. The observation of signals from both the trigonal and tetrahedral forms in spectra at pH 8.7 and 9.5 (Figure 1B,C) demonstrates that these forms are in slow exchange.

Figure 2 shows the ^{11}B NMR spectra of -boroVal as a function of pH in the presence of α-lytic protease. A strong and relatively sharp signal at –17.8 ppm is observed over the entire pH range examined (i.e., from ~4 to ~9). We assign this signal to the enzyme-bound inhibitor. A signal from the trigonal free inhibitor is also present in spectra at neutral and low pH values (Figure 2C–E) and demonstrates that free trigonal and bound tetrahedral inhibitors are in slow exchange. The presence of a signal from the trigonal inhibitor in the presence of enzyme is not surprising because the inhibitor is present in about a 2-fold molar excess over the enzyme for all spectra shown in Figure 2. The absence of evidence for the free tetrahedral inhibitor at higher pH values (Figure 2A) was however surprising. The difference in chemical shifts (–16.5 vs –17.8 ppm) and line widths (~420 vs ~135 Hz)

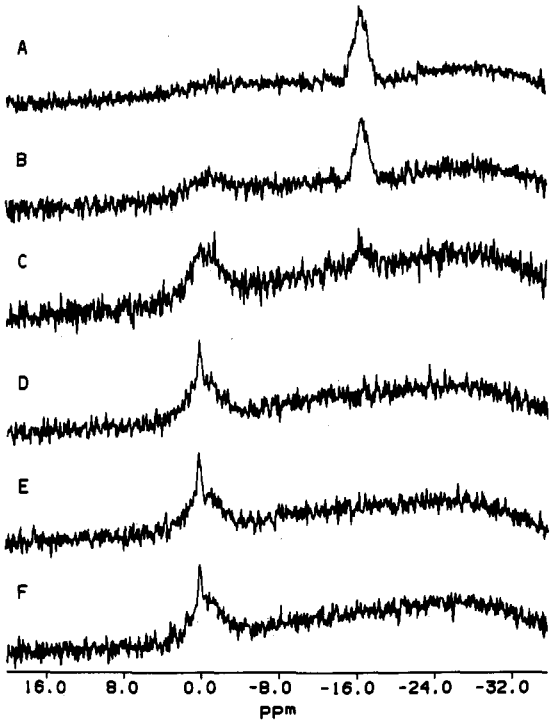

FIGURE 1: ^{11}B NMR spectra of MeOSuc-Ala-Ala-Pro-boroVal in 0.1 M KCl at (A) pH 10.2, (B) pH 9.5, (C) pH 8.7, (D) pH 7.3, (E) pH 6.1, and (F) pH 4.8. Each spectrum represents between 6000 and 8000 scans.

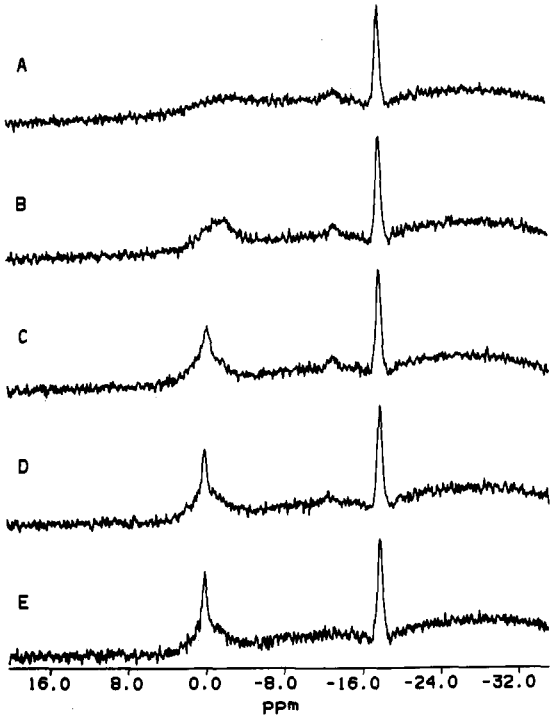

FIGURE 2: ^{11}B NMR spectra of α-lytic protease inhibited with a 2-fold molar excess of MeOSuc-Ala-Ala-Pro-boroVal in 0.1 M KCl at (A) pH 9, (B) pH 8.4, (C) pH 7.6, (D) pH 6.6, and (E) pH 4.2. Each spectrum represents between 13 000 and 15 000 scans.

between the free and bound species (Figures 1 and 2) indicates that the presence of free inhibitor should be evident in the high-pH spectra regardless of whether it is in fast, slow, or intermediate exchange with enzyme-bound inhibitor. The addition of increasing amounts of inhibitor to the solution at high pH does not increase the intensity, or change the line width and the chemical shift of the signal at –17.8 ppm (data not shown), as it should if free and enzyme-bound inhibitors

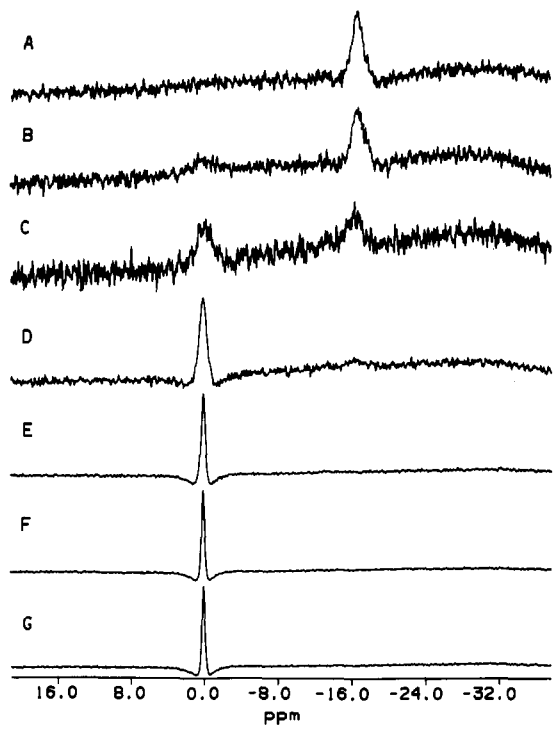

FIGURE 3: ^{11}B NMR spectra of MeOSuc-Ala-Ala-Pro-boroPhe in 0.1 M KCl at (A) pH 9.7, (B) pH 9.4, (C) pH 8.7, (D) pH 8.2, (E) pH 7.2, (F) pH 5.2, and (G) pH 4. Each spectrum represents between 5000 and 9000 scans.

were in fast or intermediate exchange. This indicates that the enzyme is saturated with inhibitor, that the exchange between free and bound inhibitor must be slow, and that for some reason the signal from the free inhibitor is not detected. The absence of a signal for the free inhibitor at high pH was not due to the presence of paramagnetic metals as the addition of EDTA did not change the NMR spectrum (data not shown).

Why is no signal detected for the free tetrahedral inhibitor? The observation of a ^{11}B signal for this species in the absence of enzyme (Figure 1A,B) suggests the presence of the enzyme must somehow render this species invisible to ^{11}B NMR detection. This conjecture was confirmed by an experiment in which about 2-fold molar excess of the inhibitor was added to a sample containing PMSF-preinactivated α-lytic protease. This sample exhibited one ^{11}B NMR signal at low pH at ~0 ppm characteristic of the free trigonal boron, but no signal characteristic of a tetrahedral boron species, either free or enzyme-bound at a high pH (data not shown). Why the presence of the protein molecule makes the free tetrahedral species invisible to ^{11}B detection is not clear and needs to be further investigated. Nevertheless, there can be no question that the ^{11}B NMR signal from the enzyme-bound inhibitor is visible and demonstrates that the boron atom is tetrahedral.

MeOSuc-Ala-Ala-Pro-boroPhe. Figure 3 shows the ^{11}B NMR spectra of -boroPhe free in solution as a function of pH. The results are very similar to those observed for free -boroVal (Figure 1). The tetrahedral species is observed at ~–16.6 ppm in high-pH spectra (Figure 3A,B), the trigonal species is seen at ~+0.1 ppm in low-pH spectra (Figure 3D–G), and the two species are in slow exchange at pH 8.7 (Figure 3C) with a transitional pK_a of about 9. There is, however, one notable difference in the boron spectra of the two inhibitors. The line width of the trigonal form of -boroPhe is much narrower (~100 Hz) than that of -boroVal (~400 Hz) although the broader signal from -boroVal inhibitor appears to have a sharp component (Figure 1D–F). An explanation for these observations will require further investigation.

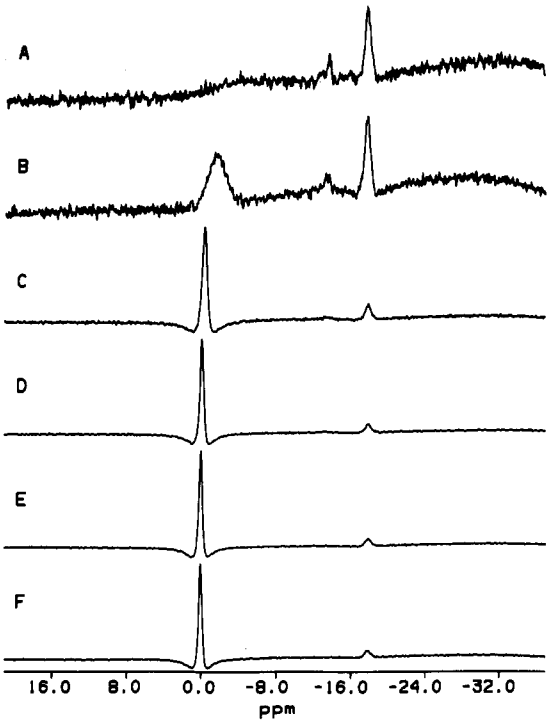

FIGURE 4: ^{11}B NMR spectra of α-lytic protease inhibited with a 2-fold molar excess of MeOSuc-Ala-Ala-Pro-boroPhe in 0.1 M KCl at (A) pH 9, (B) pH 8.5, (C) pH 7.7, (D) pH 6.9, (E) pH 5.5, and (F) pH 4.5. Each spectrum represents between 12 000 and 15 000 scans.

Figure 4 shows the ^{11}B NMR spectra of the -boroPhe inhibitor in the presence of α-lytic protease as a function of pH. The results again parallel those observed with the -boroVal inhibitor. At all pH values, the spectra show a resonance at −17.7 ppm, with a line width (~175 Hz) substantially smaller than that of the free tetrahedral inhibitor (~400 Hz). We assign this signal to the enzyme-bound inhibitor. The chemical shift, about 1 ppm upfield from the free tetrahedral form of the inhibitor and within ~0.1 ppm of that of the enzyme-bound -boroVal inhibitor, demonstrates that the boron atom in this histidine adduct complex is tetrahedral. The enzyme-bound ^{11}B signal is about the same size at all pHs. That it appears to become smaller in the spectra of Figure 4 as the pH is lowered is due to the sharpening of the signal from the free trigonal species which makes it necessary to reduce the vertical expansion in the stack plots to keep this signal on scale.

DISCUSSION

The ^{11}B NMR chemical shifts of −17.7 and −17.8 ppm for the boron atom in the -boroPhe and -boroVal complexes with α-lytic protease, respectively, about 17.9 ppm upfield from that of the free trigonal species and only about 1 ppm upfield from the free tetrahedral species, unambiguously demonstrate that the boron atom is tetrahedral in both the histidine and serine adduct complexes. The X-ray diffraction data which were interpreted as indicating a trigonal boron in the α-lytic protease complex with -boroPhe (Bone et al., 1989) therefore need to be reexamined. The fact that the boron atom has essentially the same chemical shift in the histidine and serine adduct complexes indicates the boron shift is not likely to be useful in identifying and distinguishing between histidine and serine adducts in other boronic acid inhibited enzyme complexes. This is not surprising, however, on the basis of past studies of ^{11}B NMR chemical shifts. For example, Kidd (1983) has tabulated the ^{11}B chemical shifts of a large number of compounds and has shown that the main factor determining the nuclear shielding of a boron atom, and thus its chemical shift, is the symmetry about the boron atom. A change in one of the ligating groups frequently has little or no effect on the chemical shift. Thus, it is well established that ^{11}B chemical shifts should be unambiguous in revealing the symmetry of a particular boron atom but not so useful in distinguishing between differently liganded boron functionalities with the same symmetry.

Table I: Summary of the ^{11}B Chemical Shifts of Free and Enzyme-Bound Peptide Boronic Acid Inhibitors

inhibitor	free trigonal	free tetrahedral	enzyme-bound tetrahedral	$\Delta\delta$|bound − free|
^{11}B Chemical Shifts at 160.46 MHz[a]				
MeOSuc-Ala-Ala-Pro-boroVal	0.1	−16.5	−17.8	1.3
MeOSuc-Ala-Ala-Pro-boroPhe	0.1	−16.6	−17.7[b]	1.1
			−17.3[c]	0.7
^{11}B Chemical Shifts at 64.21 MHz[d]				
MeOSuc-Ala-Ala-Pro-boroPhe	−0.6	−16.9	−23.9[c]	7

[a] Chemical shifts are in ppm from boric acid. [b] α-Lytic protease complex. [c] Chymotrypsin complex. [d] Data from Zhong et al. (1991) and recalculated to boric acid standard.

A comparison of our results with those of a previous ^{11}B NMR study of -boroPhe and its complex with chymotrypsin lead to some interesting conclusions (Zhong et al., 1991). Zhong et al. reported ^{11}B NMR chemical shifts for the free trigonal and tetrahedral forms of the -boroPhe inhibitor of −0.6 and −16.9 ppm, respectively, which is in reasonably good agreement with the shifts of +0.1 and −16.6 ppm, respectively, we observe (Table I). However, they reported a ^{11}B chemical shift for the chymotrypsin-bound inhibitor of −23.9 ppm. This chemical shift is 7 ppm upfield from the position they and we observe for the free tetrahedral species, and more than 6 ppm upfield from the shifts we observe either for the serine adduct complex of α-lytic protease with -boroVal or for the histidine adduct complex with -boroPhe. At first glance, these results may seem puzzling because there is no reason to expect that a boron atom bound as a serine adduct to chymotrypsin should experience an environment so much different from one bound as a serine adduct to α-lytic protease. If anything, we should expect a greater difference in the environment of the boron atoms between the -boroPhe and the -boroVal complexes of α-lytic protease, which respectively form histidine and serine adducts and which have nearly identical chemical shifts, than between the -boroPhe–chymotrypsin complex and the -boroVal–α-lytic protease complex, which are both serine adducts.

There is, however, an explanation for these observations. The theory describing the NMR properties of quadrupolar nuclei predicts that when the motional properties of a quadrupolar nucleus place it outside the extreme narrowing limit, i.e., when $\omega_0\tau_c \gg 1$ (where ω_0 is the spectrometer frequency and τ_c the motional correlation time), the resonance position can become dependent on the magnetic field strength, a phenomenon referred to as a second-order dynamic frequency shift (Butler & Eckert, 1989). The magnitude of the frequency shift, $\Delta\omega_d$, for spin $^3/_2$ nuclei is predicted to be inversely proportional to the magnetic field strength as shown in eq 1

$$\Delta\omega_d = (25 \times 10^{-3})\chi^2/\omega_0 \quad (1)$$

where χ is the quadrupolar coupling constant (Neurohr et al., 1983; Zhong et al., 1991). The correlation time of proteins with a molecular weight of ~20 000, such as α-lytic protease

and chymotrypsin, is typically about 1×10^{-8} s. For our ^{11}B measurements at 160.46 MHz, $\omega_0\tau_c \sim 10$, and for those of Zhong and co-workers at 64.21 MHz, $\omega_0\tau_c \sim 4$. Thus, in both studies, the motional properties of the enzyme-bound boron should be outside the extreme narrowing limit. Assuming a quadrupolar coupling constant of ~1 MHz, eq 1 predicts a second-order dynamic frequency shift of about 1 ppm at 160.46 MHz and of about 6 ppm at 64.21 MHz. Thus, eq 1 comes close to accounting for the difference in the observed shifts for enzyme-bound boron between our work and that of Zhong et al. (1991) and therefore supports the idea that the enzyme-bound boron nucleus is outside the extreme narrowing region and that we are indeed observing a second-order dynamic frequency shift.

Further support for this hypothesis comes from the ^{11}B line widths. Theory also predicts that outside of the extreme narrowing limit the boron line widths should become sharper as the correlation time or magnetic field strength increases (Butler & Eckert, 1989). Our spectra (Figures 1–4) show that the ^{11}B resonance from the enzyme-bound boron is much sharper ($\Delta\nu_{1/2} \sim$ 135–175 Hz) than that for the free tetrahedral boron ($\Delta\nu_{1/2} \sim$ 450 Hz). In addition, the line widths we observe at 160.46 MHz for the enzyme-bound boron appear to be substantially smaller that those observed by Zhong et al. (1991) at lower frequency (64.21 MHz). Another ^{11}B NMR study at an intermediate magnetic field strength (128 MHz) of an aryl boronic acid bound to chymotrypsin and subtilisin also showed that the ^{11}B NMR signal from boron bound to the enzyme was significantly sharper than that of the free tetrahedral inhibitor (Baldwin et al., 1991).

Zhong et al. (1991) speculated that the difference in chemical shift they observed between free and enzyme-bound tetrahedral boron might in part be due to a second-order dynamic frequency shift. Their data, however, were from a single magnetic field strength, and they were thus not able to establish the magnetic field dependence of the position of the bound signal. Our results at higher magnetic field together with those of Zhong et al. (1991) at lower field strongly suggest that the shift of the enzyme-bound boron signal is indeed magnetic field dependent. However, our results are with α-lytic protease while those of Zhong et al. (1991) are with chymotrypsin which raises the possibility of enzyme-mediated environmental effects as a explanation for the difference in shifts between the two studies. To rule this out, we have examined the -boroPhe–chymotrypsin complex, examined by Zhong et al. (1991) at 64.21 MHz, at 160.46 MHz. As shown in Table I, at 160.46 MHz, the enzyme-bound boron atom in this complex resonates at –17.3 ppm, which is nearly the same position as that of the peptide boronic acid inhibitor complexes of α-lytic protease, and is about 7 ppm downfield from its reported position in the 64.21-MHz spectra. This result eliminates enzyme-mediated environmental effects as an explanation for the differing chemical shifts and confirms the magnetic field dependence of the ^{11}B NMR chemical shifts of enzyme-bound boronic acid inhibitors. The agreement between the $\Delta\omega_0$ predicted by eq 1 and experimentally observed chemical shifts as a function of magnetic field indicates that most if not all of the difference observed between the chemical shifts of free and enzyme-bound tetrahedral boron, at both magnetic fields, can be attributed to second-order dynamic frequency shifts.

To summarize, the ^{11}B chemical shifts unambiguously show that the boron atom is tetrahedral in both histidine and serine adducts. At 160.46 MHz, the signal of the enzyme-bound inhibitors occurs about 1 ppm upfield from, and is substantially narrower than, the ^{11}B signal from the free tetrahedral inhibitors. However, at 64.21 MHz, the enzyme-bound boron signal is broader and occurs about 7 ppm upfield (Zhong et al., 1991) from its position in 160.46-MHz spectra. The frequency dependency of the ^{11}B line widths and chemical shifts indicates that the motional properties of the enzyme-bound ^{11}B nucleus place it outside of the extreme narrowing region and therefore that the difference in resonance frequency between the free and enzyme-bound tetrahedral boron atoms, at both magnetic fields, can largely be attributed to a second-order dynamic frequency shift. The observation that the ^{11}B NMR line width becomes narrower as the correlation time and magnetic field strength increase demonstrates that ^{11}B NMR spectroscopy should become more amenable as the size of the molecule under study becomes larger rather than smaller, as is the case for spin $^1/_2$ nuclei such as ^{1}H.

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