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# $^{11}$B NMR STUDIES OF AN ARYL BORONIC ACID BOUND TO CHYMOTRYPSIN AND SUBTILISIN

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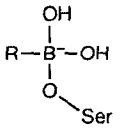
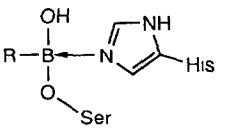
**Summary**:The binding of aryl boronic acids to α-chymotrypsin and subtilisin has been studied by $^{11}$B NMR spectroscopy; evidence is provided for the direct spectroscopic observation of a tetrahedral enzyme/boronate complex.

Boronic acids are well-known reversible inhibitors of serine proteases. Their inhibitory action has been ascribed to a reaction with an active-site serine residue, to form a tetrahedral boronate complex which mimics the putative tetrahedral intermediate formed during substrate hydrolysis.[1] Recent $^{15}$N NMR[2] and X-ray crystallography[3] studies, however, have provided evidence for at least two distinct types of covalent boronate/protease complex. In addition to the anticipated covalent, tetrahedral boronate adduct (**1**), bound to the active-site serine, a second structural type was observed. This was a so called trigonal adduct (**2**), also covalently bound to the serine, but with a histidine residue forming a fourth coordinate bond. The factors responsible for the formation of these different boronate/enzyme structures are at present unclear and are of current interest.[4]




**1**




**2**

A potential method that could lead to a rapid assessment of the bonding environment around the boron nucleus is the observation of the putative enzyme-bound complex using $^{11}$B NMR spectroscopy. Recently, Adebodun and Jordan have reported $^{11}$B NMR studies of phenylboronic acid (PBA) and 3,5-bis(trifluoromethyl)phenylboronic acid (BTFPBA) bound to α-chymotrypsin and subtilisin.[5,6] In the case of PBA [5] the spectra they obtained were interpreted as the result of a fast-exchange average between free boronic acid and an enzyme-bound boronate adduct. Titration of the boronic acids with increasing amounts of enzyme and monitoring the changes in the $^{11}$B spectra allowed extrapolated estimates of the chemical shifts, linewidths and quadrupole coupling constants for the enzyme-bound species. From these values, the bound species was assigned as a tetrahedral boronate adduct of broad $^{11}$B linewidth (1.9 x $10^3$ Hz) and a *calculated* chemical shift value of -12.7ppm. In a related study similar calculations led to a predicted value of -12.3ppm for the $^{11}$B NMR signal resulting from the complex formed between BTFPBA and subtilisin.[6] Herein, we report the results of our own studies, using BTFPBA, in which evidence is presented for the first direct spectroscopic observation of a tetrahedral boronate/protease species by $^{11}$B NMR spectroscopy.

BTFPBA is a potent inhibitor of subtilisin ($K_i$=5.3 x $10^{-6}$) and chymotrypsin displaying optimal binding around pH 7.5[7]. Initially we examined the $^{11}B$ spectroscopy of BTFPBA itself in order to acquire reference spectra for comparison with any enzyme bound species.[8] The strongly electron withdrawing 3,5-bis(trifluoromethyl)phenyl group lowers the pKa[10] of BTFPBA to around 7.0. Acidic solutions (pH <2) of BTFPBA were found to exhibit a single $^{11}B$ chemical shift of 10.0 ppm ( all chemical shifts were referenced against external $B(OMe)_3$ at 0.0ppm) consistent with the preponderance of a trigonal species at low pH. When the pH was raised to >12 a $^{11}B$ chemical shift of -15.7 ppm was observed, indicative of the formation of a tetrahedral species. At pH 7.4, fresh samples were found to display a single peak at -8.0 ppm resulting from the fast-exchange averaging of the two boronic acid species. On standing at pH 7.4 a new peak at 0.7 ppm became apparent, which was subsequently shown, by doping with authentic material, to be boric acid resulting from slow hydrolysis of the BTFPBA.[11]

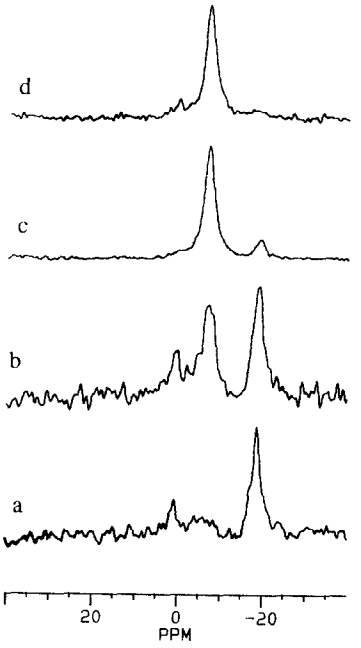



Figure 1.

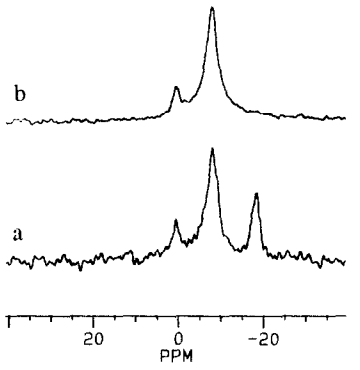



Figure 2.

**Figure 1.** *$^{11}B$ NMR of 0.4 mM α-chymotrypsin and (a) 0 2 mM BTFPBA; (b) 0.4 mM BTFPBA; (c) 1.0 mM BTFPBA; and of (d) 0.4 mM PMS-chymotrypsin and 0.4 mM BTFPBA, in 50 mM sodium phosphate, pH 7.4 at 23°C.*

**Figure 2.** *$^{11}B$ NMR of (a) 0.4 mM subtilisin and 0.4 mM BTFPBA; (b) 0.4 mM PMS-subtilisin and 0 4 mM BTFPBA; in 50 mM sodium phosphate, pH 7.4 at 23°C.*

When solutions of 0.4 mM α-chymotrypsin at pH 7.4 were treated with either 0.2 mM, 0.4 mM or 1.0 mM BTFPBA (Figures 1a,b and c, respectively), three signals were observed; a peak at -8.0 ppm, corresponding to the fast-exchange average of free trigonal and free tetrahedral BTFPBA; a small peak at 0.7 ppm corresponding to boric acid and a peak at -18.7 ppm (linewidth = 200 Hz)whose relative intensity was shown to increase proportionally with the fraction of chymotrypsin present. The peak at -18.7 ppm is in the range that has been reported to be highly characteristic of a tetrahedral boronate species[12]. It was completely removed by the addition of three molar equivalents of phenylmethylsulphonyl fluoride (PMSF) and was absent in the spectra of control samples using chymotrypsin which had been inactivated with PMSF prior to the addition of BTFPBA (Figure 1d). A similar series of experiments were performed using subtilisin instead of chymotrypsin and an analogous set of $^{11}B$ spectra were obtained (chemical shift of the putative tetrahedral species was found to be -18.4ppm, linewidth= 220 Hz) (Figure 2).

These results present strong evidence that, under our conditions, the exchange between trigonal BTFPBA and its tetrahedral α-chymotrypsin and subtilisin adducts is probably a slow exchange process at room temperature ($k < 3 \times 10^3\ s^{-1}$), and that discrete signals for the enzyme bound tetrahedral complexes can be observed by $^{11}B$ NMR. Moreover, the linewidths of the bound species were remarkably narrow for an enzyme bound species, possibly due in part to the smaller boron quadrupole coupling constants inherent with the more symmetrical tetrahedral boron environments. The discrepancies between our *observed* chemical shift and linewidth values for the boronate/enzyme complexes and those previously obtained by *calculation* [5,6] are currently under investigation.

Crystallographic studies of the binding of aryl and alkyl boronic acids to α-chymotrypsin or subtilisin have indicated the tetrahedral adduct is covalently bound to the active-site serine residue, as in structure **1**[13]. However, certain boronic acid-serine protease complexes have been shown to involve histidine coordination, as in structure **2**[2,3]. We are currently exploring the potential of $^{11}B$ NMR as a method of differentiating between these possible enzyme/boronate adduct structures.

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8. The $^{11}B$ spectra were acquired at 128 MHz on a Bruker MSL 400 instrument using a static wide line probe and non glass sample containers. A typical spectrum was acquired with a spectral width of 50KHz, using 800 data points zero-filled to 8K and an acquisition time of 8 ms. A line broadening of 50 Hz was used for all spectra and 0.3-1 x $10^6$ scans were accumulated. The α-chymotrypsin (3 x recrystallised) and subtilisin (Carlsberg) were purchased from the Sigma Chemical Company. Irreversibly inactivated enzyme was obtained by incubation with three molar equivalents of phenylmethylsulphonyl fluoride which has been shown to covalently bind to the active site serine[9].

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10. Boronic acids are thought of as Lewis acids and thus pKa refers to the transition
$RB(OH)_2 + 2H_2O \rightleftharpoons RB(OH)_3^- + H_3O^+$.

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