Direct Observation of a Tetrahedral Boronic Acid–β-Lactamase Complex Using ^{11}B NMR Spectroscopy

Jack E. Baldwin,*[a] Timothy D. W. Claridge,[a] Andrew E. Derome,[a] Bradley D. Smith,[a] Mark Twyman[b] and Stephen G. Waley[c]

[a] The Dyson Perrins Laboratory, [b] Inorganic Chemistry Laboratory, [c] Laboratory of Molecular Biophysics and The Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK

A tetrahedral boronic acid–β-lactamase complex formed by treatment of the P99 β-lactamase enzyme from *Enterobacter cloacae* with 3-dansylamidophenylboronic acid has been directly observed using ^{11}B NMR spectroscopy.

Boronic acids have been shown to be reversible inhibitors of active-site serine β-lactamases (class A and C β-lactamases using the molecular sequence classification).[1,2] They are virtually the only active-site inhibitors of β-lactamases that are not themselves β-lactams and may be useful clinically or in mechanistic studies. All boronic acids so far examined display some degree of activity as enzyme inhibitors. They are thought to act by mimicking the high energy tetrahedral intermediate formed during β-lactam hydrolysis. The kinetics of inhibition, studied at low temperatures and by rapid reaction techniques at ordinary temperatures, show evidence for a two step mechanism for binding in which a rapid equilibrium precedes a rate-determining step. It was suggested that this slow step corresponds to a change in enzyme conformation as well as the formation of a covalent, tetrahedral complex, although there was no direct evidence for either process.[2]

Boronic acids may interact with β-lactamases in the same way as they do with the serine protease enzymes. The proteases are currently under extensive investigation and a combination of kinetic, NMR and crystallographic studies have suggested at least two types of binding, depending upon substrate structure.[3] In some cases there is clear evidence for a covalent, tetrahedral boronate complex with the active-site serine, whereas in other examples, the boronic acid forms a trigonal adduct with the serine and the active-site histidine forms a fourth coordinate bond. Although the β-lactamases resemble the serine proteases in the deployment of an active-site serine, they differ in that the cooperating residues appear to be lysine and glutamic acid (or for class C β-lactamases perhaps tyrosine) rather than histidine and aspartic acid.[4]

Here we report that using ^{11}B NMR we have been able to observe directly an enzyme-bound, tetrahedral β-lactamase–boronic acid complex. We have investigated the interaction between 3-dansylamidophenylboronic acid (DnsPBA)† and the class C β-lactamase P99 from *Enterobacter cloacae*. DnsPBA has been shown to inhibit completely the β-lactam hydrolysis action of the P99 enzyme and a dissociation constant of 2 μmol dm^{-3} for the binary complex has been determined from fluorescence measurements.[5] The ^{11}B NMR spectra of fresh samples of DnsPBA in buffer solution (50 mmol dm^{-3} sodium phosphate, pH 7.4) at concentrations above about 1.0 mmol dm^{-3}, initially displayed a single resonance at δ 8.2 [all ^{11}B spectra referenced against external $B(OMe)_3$ at δ 0.0] characteristic of the trigonal‡ boronic acid [Fig. 1(*a*)]. When the sample was allowed to stand overnight before the spectrum was acquired, a small, second peak at δ 0.7 was also observed, which was assigned as boric acid

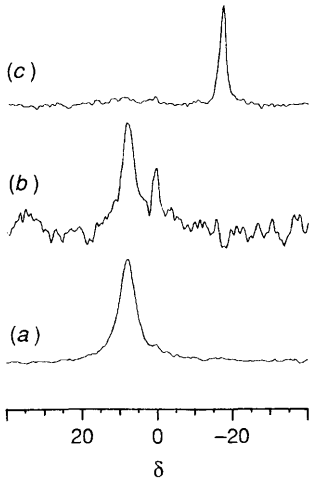

Fig. 1 ^{11}B NMR spectra of (*a*) 1.0 mmol dm^{-3} DnsPBA; (*b*) 0.1 mmol dm^{-3} DnsPBA; (*c*) 0.25 mmol dm^{-3} DnsPBA and 0.25 mmol dm^{-3} P99. All samples in 50 mmol dm^{-3} sodium phosphate, pH 7.4 and 23 °C. The ^{11}B spectra were acquired at 128 MHz on a Bruker MSL 400 instrument using a static wide-line probe (70 mm diameter) and non-glass sample containers (5 mm diameter) which were necessary for background-free spectra at low boron concentrations. A typical spectrum was acquired with a spectral width of 50 kHz, using 800 data points zero-filled to 8 K, and an acquisition time of 8 ms. Generally, a line-broadening of 100 Hz was employed and 1–4 × 10^6 scans were accumulated, for total acquisition times of 3–12 h per spectrum.

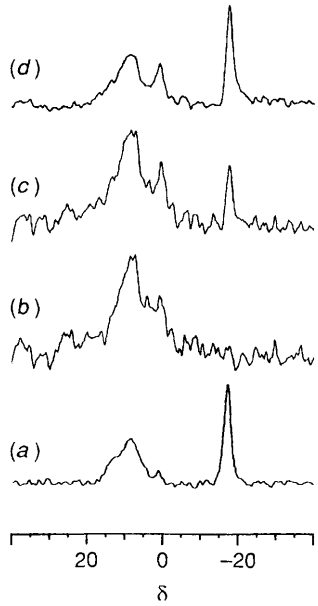

Fig. 2 ^{11}B NMR spectrum of (*a*) 1.0 mmol dm^{-3} DnsPBA and 0.25 mmol dm^{-3} P99 in 50 mmol dm^{-3} sodium phosphate, pH 7.4 and 23 °C. Carbenicillin (50 mmol dm^{-3}) was then added and spectra were acquired for (*b*) fifty minutes; (*c*) two hours and (*d*) six hours after the addition.

† *N*-(5-Dimethylamino-1-naphthylsulphonyl)-3-aminobenzeneboronic acid.

‡ Boronic acids are usually thought of as Lewis acids rather than protic acids. The pK_a for the transition $RB(OH)_2 + 2H_2O \rightleftharpoons RB(OH)_3^- + H_3O^+$ is about 8.8 for this boronic acid, therefore, at pH 7.4 there is a large preponderance of trigonal species.

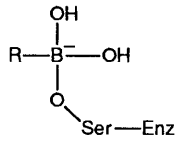

Fig. 3 The tetrahedral boronate at the enzyme active site

resulting from the hydrolysis of the DnsPBA.[6]§ Spectra of aqueous DnsPBA at concentrations below about 0.5 mmol dm^{-3} (overnight acquisition needed) could never be collected without the presence of this hydrolysis byproduct [Fig. 1(*b*)]. Treatment of a 0.25 mmol dm^{-3} solution of DnsPBA with an equimolar amount of P99 enzyme resulted in the disappearance of these two peaks and the appearance of an upfield peak at δ −17.4 [Fig. 1(*c*)]. This chemical shift is highly characteristic of a tetrahedral boronate species[7] and hence was assigned as the enzyme-bound, tetrahedral complex. Furthermore, there was a significant difference between the linewidths for the free (580 Hz) and the bound (160 Hz) resonances which we attribute to the change from the trigonal to a more symmetrical tetrahedral environment which produces a reduction in the quadrupolar broadening of the bound resonance. The conversion to bound complex was apparently stoichiometric, in agreement with the known K_d of 2μmol dm^{-3}.

Evidence that the interaction of the boronic acid with the enzyme is specifically at the active site, was obtained from the following experiments. A solution of 1.0 mmol dm^{-3} DnsPBA and 0.25 mmol dm^{-3} P99 [^{11}B spectrum shown in Fig. 2(*a*)] was treated with 50 mmol dm^{-3} of carbenicillin which is known to be a specific but sluggish substrate for this enzyme due to a slow, rate-limiting deacylation step ($k_{cat} = 3 \times 10^{-3}$ s^{-1}. K_M = 5–11 nmol dm^{-3}).[8] ^{11}B acquisition for 50 min after carbenicillin addition [Fig. 2(*b*)] showed complete loss of the peak at δ −17.4 and restoration of the signals at δ 8.2 and 0.7, implying that the boronic acid had been displaced from the active site by the tighter binding carbenicillin. Acquisition for two hours [Fig. 2(*c*)] and for six hours [Fig. 2(*d*)] after carbenicillin addition shows the signal at δ −17.4 beginning to reappear as the carbenicillin is consumed and the enzyme active-site serine becomes available again for occupation by the DnsPBA. In a similar experiment, 0.25 mmol dm^{-3} of P99 was first treated with 1.25 mmol dm^{-3} of 6-β-iodopenicillanic acid, a potent irreversible inhibitor of the enzyme known to react covalently with the active-site serine,[9] and then 0.25 mmol dm^{-3} DnsPBA was added. The resulting ^{11}B spectrum showed complete absence of the tetrahedral species at δ −17.4, even after eight hours of acquisition, in agreement with the irreversible nature of the inhibition.

In conclusion, we have demonstrated direct NMR evidence for a tetrahedral, boronic acid–β-lactamase complex, most likely covalently bound *via* the enzyme active-site serine residue as indicated in Fig. 3. The apparent rate of exchange between free and bound boronic acid is slow on the ^{11}B NMR timescale and allows discrete observation of both species.

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§ The products from hydrolysis of DnsPBA, namely boric acid and 3-dansylamidobenzene, were identified by spectral and chemical analysis. In anhydrous organic solutions the hydrolysis process was not observed.

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