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β-Lactamase inhibitors

The inhibition of serine β-lactamases by specific boronic acids

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Many β-lactamases have active-site serine residues, and are competitively inhibited by boronic acids. Hitherto, the boronic acids used have lacked any structural resemblance to the substrates of β-lactamases. Phenylacetamidomethaneboronic acid, trifluoroacetamidomethaneboronic acid and 2,6-dimethoxybenzamidomethaneboronic acid have now been synthesized. The first of these contains the side-chain moiety of penicillin G, and the last that of methicillin. The pH-dependence of binding of the first inhibitor to β-lactamase I from *Bacillus cereus* revealed p*K* values of 4.7 and 8.2 for (presumably) active-site groups in the enzyme. The kinetics of inhibition were studied by cryoenzymology and by stopped-flow spectrophotometry. These techniques provided evidence for a two-step mechanism of binding of the first two boronic acids mentioned above to β-lactamase I, and for benzeneboronic acid to a β-lactamase from *Pseudomonas aeruginosa*. The slower step is probably associated with a change in enzyme conformation as well as the formation of an O–B bond between the active-site serine hydroxy group and the boronic acid.

INTRODUCTION

β-Lactamases are mechanistically interesting and clinically important enzymes. Their clinical importance stems from being a major cause of resistance of bacterial pathogens to the widely used β-lactam antibiotics. The mechanistic interest of β-lactamases derives from their resemblance to the serine proteinases in the deployment of an active-site serine residue, but differing in that the co-operating residues appear to be lysine and glutamic acid, rather than histidine and aspartic acid (Herzberg & Moult, 1987). Again, β-lactamases resemble proteinases in catalysing the hydrolysis of an amide bond, but differ in that the basic residue has to be deprotonated in proteinases but (apparently) protonated in β-lactamases (Herzberg & Moult, 1987).

Inhibitors of β-lactamases may be useful clinically and for crystallographic (or n.m.r.) studies. Boronic acids occupy a special place among β-lactamase inhibitors. They are virtually the only active-site-directed inhibitors of β-lactamases that are not themselves β-lactams. β-Lactams have drawbacks for crystallographic studies, as they are usually hydrolysed and the products will diffuse away, but boronic acids are, in effect, stable inhibitors, and will remain bound at the active site. Moreover, boronic acids that can diffuse through the outer membane are conceivably clinically useful β-lactamase inhibitors.

The boronic acids that have hitherto been studied as β-lactamase inhibitors (Dobozy *et al.*, 1971; Kiener & Waley, 1978; Beesley *et al.*, 1983) owe their affinity largely to the boronic acid group, and lack an appropriate side chain. We now report the synthesis of phenylacetamidomethaneboronic acid (BzB) (I) and the related compounds trifluoroacetamidomethaneboronic acid (TFB) (II) and 2,6-dimethoxybenzamidomethaneboronic acid (MeB) (III), and their interaction with the class A β-lactamase I from *Bacillus cereus*. The use of low-temperature techniques, or rapid reaction methods at ordinary temperatures, has enabled us to detect two-step binding. The marked effects of pH on binding have also been characterized. The interaction of benzeneboronic acid (PhB) with a class C β-lactamase also takes place by a two-step mechanism. Our results confirm the potential usefulness of boronic acids in structural studies of β-lactamase action.

(I) $Ph–CH_2–CONH–CH_2–B(OH)_2$
(II) $CF_3–CH_2–CONH–CH_2–B(OH)_2$
(III) $2,6\text{-}(MeO)_2C_6H_3–CONH–CH_2–B(OH)_2$

MATERIALS AND METHODS

β-Lactamase I from *Bacillus cereus* 569H/9 was prepared as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980). The use of a Millipore Pellikon cassette system to 'desalt' and concentrate the eluate from Celite greatly speeded up the preparation. β-Lactamase from the constitutive mutant of *Pseudomonas aeruginosa* 1822 S/H (Flett *et al.*, 1976; Berks *et al.*, 1982) was prepared by affinity chromatography (Cartwright & Waley, 1984).

N.m.r. spectra were recorded on a Bruker AM250 or AM500 spectrometer. ^1H- and ^{13}C-n.m.r. chemical shifts are in p.p.m. downfield from external tetramethylsilane ([^2H]chloroform) as reference. ^{11}B-n.m.r. chemical shifts are in p.p.m. downfield from external boron trifluoride ([^2H]chloroform) as reference.

Mass spectra were recorded on a V. G. Micromass 30FD spectrometer under electron-impact (e.i.) or ammonia chemical-ionization (NH_3 c.i.) conditions.

Abbreviations used: BzB, phenylacetamidomethaneboronic acid; TFB, trifluoroacetamidomethaneboronic acid; MeB, 2,6-dimethoxybenzamidomethaneboronic acid; PhB, benzeneboronic acid.

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Preparation of acylaminomethaneboronic acids

Dibutyl iodomethaneboronate is a common intermediate for the preparation of acylaminomethaneboronic acids either by the method of Linquist & Nguyen (1977) or by that of Matteson *et al.* (1981). It was prepared via iodomethylmercuric iodide by the method of Matteson & Cheng (1968), and had δ_B ([^{2}H]-chloroform) 28.0 p.p.m and showed characteristic broadening of the CH_2B peak in the ^{1}H-n.m.r. spectrum due to ^{11}B quadrupolar scalar relaxation.

Attempts to get dibutyl iodomethane boronate to react with phenylacetamide by the method of Linquist & Nguyen (1977) were unsuccessful. It was therefore instead converted into dibutyl *NN*-bis(trimethylsilyl)-aminomethaneboronate by the method of Matteson *et al.* (1981).

(i) Phenylacetamidomethaneboronic acid (BzB) (I). Dibutyl *NN*-bis(trimethylsilyl)aminomethaneboronate (194 mg, 0.59 mmol) was vigorously stirred during addition of phenylacetyl chloride (0.60 ml, 5.0 mmol) at −78 °C. The solution was allowed to warm to 20 °C over 2 h; then, after cooling to −78 °C, methanol (2 ml, 50 mmol) was added. After 30 min at 20 °C, water (10 ml) was added and the whole was stirred for 16 h (pH 1). Washing with diethyl ether (3 × 15 ml) and removal of water left a clear oil (66 mg, 59%). N.m.r.: δ_H (^2H_2O) 500 MHz 7.15 (5H, m, C_6H_5), 3.53 (2H, s, CH_2Ph) and 2.20 p.p.m. (2H, s, CH_2B); δ_C (^2H_2O) 250 MHz 176.7 (s, *C*=O), 129–127 (aromatic), 38.5 (t, CH_2Ph) and 32.5 p.p.m. (v. broad, CH_2B); δ_B (^2H_2O) 80 MHz 18.1 p.p.m. (s). M.s.: *m/z* (NH_3 c.i.) 150 ($PhCH_2$–$CONH_2$–CH_2^+).

(ii) Trifluoroacetamidomethaneboronic acid (TFB) (II). Dibutyl *NN*-bis(trimethylsilyl)aminomethaneboronate (0.5 ml, 1.3 mmol) in dichloromethane (1 ml) was vigorously stirred during addition of trifluoroacetic anhydride (2.0 ml, 14.0 mmol) at −78 °C. The solution was allowed to warm to 20 °C over 2 h, and evaporated; then, after cooling to −78 °C, methanol (2 ml, 50 mmol) was added. After 30 min at 20 °C, water (10 ml) was added and the whole was stirred for 2 h (pH1). Washing with diethyl ether (3 × 15 ml) and freeze-drying left a white powder (61 mg, 27%). N.m.r.: δ_H (^2H_2O) 250 MHz 2.77 p.p.m. (2H, s, CH_2B) δ_B (^2H_2O) 80 MHz 29.1 p.p.m. (s).

(iii) 2,6-Dimethoxybenzamidomethaneboronic acid (MeB) (III). Dibutyl *NN*-bis(trimethylsilyl)aminomethaneboronate (0.5 ml, 1.3 mmol) in dichloromethane (1 ml) was vigorously stirred during addition of 2,6-dimethoxybenzoyl chloride (1.5 g, 7.5 mmol) in dichloromethane (3 ml) at −78 °C. The solution was allowed to warm to 20 °C over 2 h, and evaporated; then, after cooling to −78 °C, methanol (2 ml, 50 mmol) was added. After 30 min at 20 °C, water (10 ml) was added and the whole was stirred for 2 h (pH 1). Washing with diethyl ether (3 × 15 ml) and freeze-drying left a white solid (121 mg, 39%). N.m.r.: δ_H (^2H_2O) 250 MHz 6.7 (3H, m, C_6H_3), 3.75 (6H, s, $2 \times OCH_3$) and 2.70 p.p.m. (2H, s, CH_2B); δ_B (^2H_2O/[$^2H_4$]methanol) 80 MHz 28.7 p.p.m. (s); δ_C (^2H_2O/[$^2H_4$]methanol) 63 MHz 171.2 (*C*=O), 160–105 (aromatics, 4 signals), 56.9 (OCH_3) and 33.0 p.p.m. (broad, CH_2B). M.s.: *m/z* (e.i.) 267 (M^+).

Measurement of β-lactamase activity

The activity of β-lactamase I was measured with nitrocefin (O'Callaghan *et al.*, 1972) as substrate by the change in absorbance at 500–580 nm, with either a Cary 219 or a Cecil CE 373 spectrophotometer. The activity of the *Pseudomonas* β-lactamase was measured with cephalosporin C [recrystallized as described by Newton & Abraham (1956)] as substrate, the change in absorbance at 260–280 nm being recorded.

Cryoenzymology

The apparatus was as described previously (Bicknell & Waley, 1985); solutions were mixed by stirring slowly by hand for 1 min with a plastic rod of square cross-section. The volume of added solution containing enzyme was no greater than 20 μl, and the temperature had returned to its original value within 3 min. The inhibition of β-lactamase I by boronic acids, with nitrocefin (25–50 μM) as substrate, at −30 °C was studied with 50%-saturated ammonium acetate (the solution saturated at 4 °C was diluted with an equal volume of water) (Cartwright & Waley, 1987). The *Pseudomonas* β-lactamase was added as a solution in 50% (v/v) ethylene glycol to the substrate in 40% (v/v) dimethyl sulphoxide, the enzyme being insufficiently soluble in the latter mixture. The concentration of the substrate (cephalosporin C) was 1 mM, about $0.6 \times K_m$.

The enzymes were shown to be stable during catalysis by the method of Selwyn (1965).

Stopped-flow spectrophotometry

Experiments were carried out at 25 °C in a Hi-Tech SF-42 (Hi-Tech Scientific, Salisbury, Wilts., U.K.) instrument, with automatic data acquisition. Use of signal averaging to improve the ratio of signal to noise was important in several experiments. The hydrolysis of cephalosporin C (40 μM, about $0.8 \times K_m$) by the *Pseudomonas* β-lactamase was carried out in 0.1 M-NaCl/20 mM-Mops/NaOH buffer, pH 7. The hydrolysis of nitrocefin (50 μM, about $0.7 \times K_m$) by β-lactamase I was carried out in 0.5 M-NaCl/20 mM-Mes/NaOH buffer, pH 6.

pH-dependence of the inhibition constant

These experiments with β-lactamase I were carried out at 30 °C in the following buffers, in 0.5 M-NaCl: Mes/NaOH (pH 5–6.5), Mops/NaOH (pH 6.5–8) and triethanolamine/HCl (pH 8–9). Initial rates were determined from progress curves (Duggleby, 1985) and fitted to Hanes plots to obtain $K_{m(app.)}/k_{cat.}$. Parallel plots in the presence or absence of inhibitor confirmed that inhibition was competitive.

RESULTS

pH-dependence of inhibition of β-lactamase by boronic acids

Inhibition constants for the effect of BzB (I) on the hydrolysis of nitrocefin by β-lactamase I were determined as follows. Progress curves were used to obtain initial rates by the method of Duggleby (1985), in which non-linear regression is used, with the final absorbance being treated as an unknown parameter. The non-linear regression was again used to fit the data to the Michaelis–Menten equation; the program of Duggleby

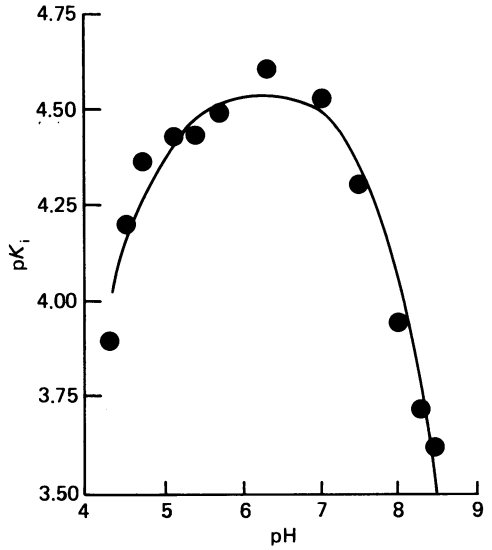
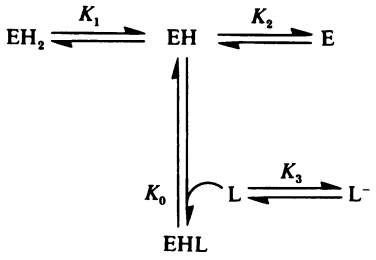


Fig. 1. Variation of pK_i with pH for BzB (I) and β-lactamase I

The reactions were carried out in 0.5 M-NaCl at 30 °C, with nitrocefin as substrate.



Scheme 1. Model for the pH-dependence of inhibition by boronic acids

(1984) was used for both regressions. The standard errors were lower for $K_m/k_{cat.}$ than for K_m, and so it was better to use the former to obtain K_i, from:

$$K_i = C_0[I]/(C-C_0)$$

where C and C_0 are the values of $K_m/k_{cat.}$ found in the presence and in the absence of inhibitor respectively and [I] is the concentration of inhibitor.

The plot of pK_i against pH shows (*a*) that there is a marked pH-dependence, (*b*) a pH optimum in approximately neutral solution, and (*c*) that the curve is not symmetrical (Fig. 1). The inhibitor has a measured pK_a of 8.1, under conditions used for the kinetics. The pK_a of the inhibitor is superimposed on the effects of the ionization of the enzyme. The results were interpreted on the basis of Scheme 1, which leads (with the usual assumptions) to:

$$pK_i = pK_0 - \log(1+10^{pH-pK_2}+10^{pK_1-pH}) - \log(1+10^{pH-pK_3}) \quad (1)$$

The ionization of boric acid and boronic acids takes place as follows:

$$RB(OH)_2 + 2H_2O \rightleftharpoons R\bar{B}(OH_3) + H_3O^+ \quad (2)$$

and so is shown as $L \rightleftharpoons L^-$ in Scheme 1. The data were fitted to eqn. (1) by the PATTENSEARCH program of R. J. Beynon (Department of Biochemistry, University of Liverpool, Liverpool, U.K.), with pK_3 (the ionization of the boronic acid) = 8.1, giving pK_1 4.7, pK_2 8.2 and pK_0 4.5. The values of pK_1 and pK_2 are probably sufficiently close to the values of 4.85 and 8.6 respectively obtained from the pH-dependence of $k_{cat.}/K_m$ in the hydrolysis of penicillin G (Waley, 1975) to suggest that the same ionizations are being detected.

The curve in Fig. 1 has effectively zero slope at pH 6.5. The slope of such a plot gives the number of protons given off (or taken up) when the ligand binds (Laskowski & Finkenstadt, 1972). Hence there is no proton given off at pH 6.5 when the boronic acid binds. This point was confirmed by a direct experiment in which ligand binding was carried out in the presence of indicator. If the boronic acid formed a covalent bond with the active-site serine residue, a reaction analogous to the ionization shown in eqn. (2), then a proton would be evolved, unless it were taken up by another group. Active-site residues that are a candidate for such a group are Lys-73 and (perhaps) Glu-166. When PhB binds to subtilisin a covalent bond is formed (Matthews *et al.*, 1975), but there is no proton evolved (at pH 8) (Philipp & Bender, 1971; Nakatani *et al.*, 1975). A histidine residue is a probable site for proton acceptance in subtilisin, but β-lactamase I lacks an active-site histidine residue.

Scheme 1 does not show E (the deprotonated form of the enzyme) binding to L. When the ionization $EHL \rightleftharpoons EL^- + H^+$ (with ionization constant K_4) was taken into account by including a term $-\log(1+10^{pH-pK_4})$ in eqn. (1), the value of pK_4 found was 28. Such a high value implies that this ionization is not significant and so the combination of E and L can be disregarded.

The information from pH-dependence of binding is, of course, thermodynamic, and, although it specified the composition of species, for information about the mechanism by which the species are formed kinetic data are required.

Kinetics of inhibition of β-lactamases by boronic acids

There was no sufficiently marked spectrosopic change (u.v. or fluorescence) in β-lactamase I on interaction with the boronic acids, and so the interaction was necessarily studied by utilizing the boronic acid as inhibitor. The inhibition was competitive, and progress curves under the usual conditions showed no unusual features. At low temperatures, however, biphasic progress curves were observed (Fig. 2); by lowering the temperature, we have induced slow-binding inhibition (Morrison, 1982). The analysis of such curves is now described. The curves were fitted to eqn. (3) (Cha, 1975):

$$p = v_f t + (v_0 - v_f)(1-e^{-kt})/k + d \quad (3)$$

where p is the concentration of product at time t, v_0 and v_f are the original and final rates respectively, k is the first-order apparent rate constant characterizing the transition to a linear rate, and d is a parameter to correct for uncertainty in the start of the reaction. An alternative method of correction is to measure the changes in concentration of product (y) and time (x) from an arbitrary origin at time t_0, when it may readily be shown that:

$$y = v_f x + (v_0 - v_f)e^{-kt_0}(1-e^{-kx})/k \quad (4)$$

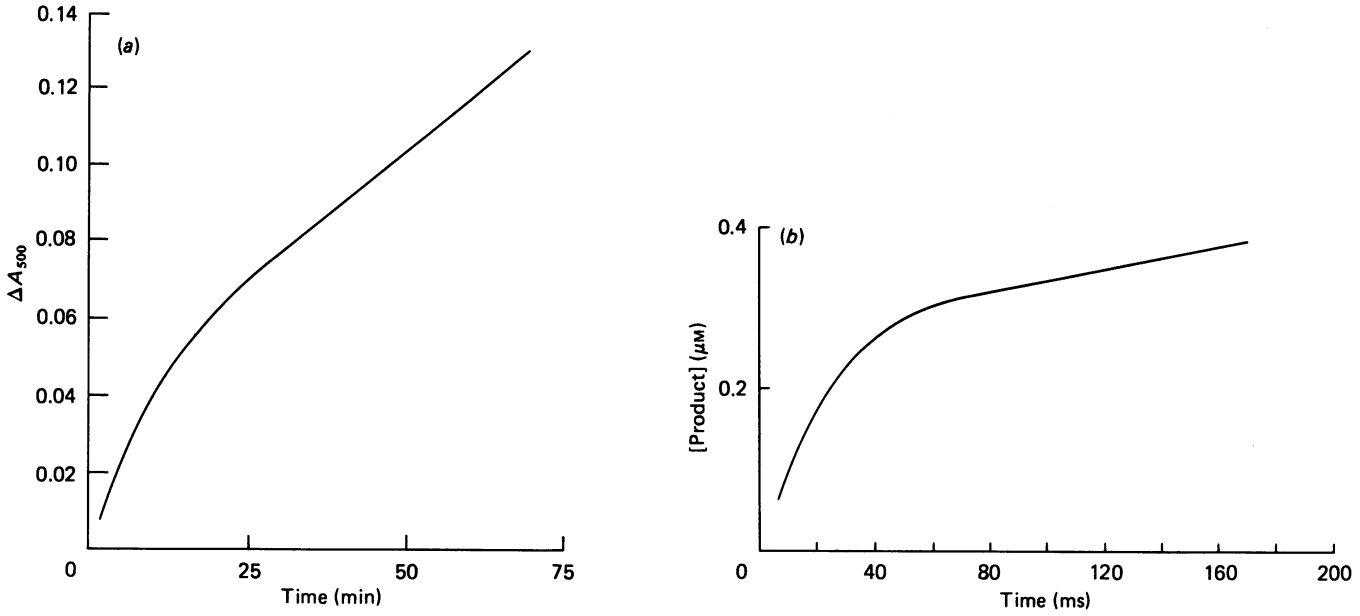


Fig. 2. Biphasic progress curves from the hydrolysis of nitrocefin by β-lactamase I in the presence of BzB (I)

(*a*) The reaction was carried out in 50%-saturated ammonium acetate at −30 °C; the concentrations of substrate, enzyme and inhibitor were 50 μM, 0.125 μM and 8 μM respectively. (*b*) The reaction was carried out at 25 °C in the stopped-flow spectrophotometer; the concentrations of substrate, enzyme and inhibitor were 50 μM, 5 μM and 500 μM respectively in 0.5 M-NaCl/20 mM-Mes buffer, pH 6. Product concentration was calculated from the observed trace.

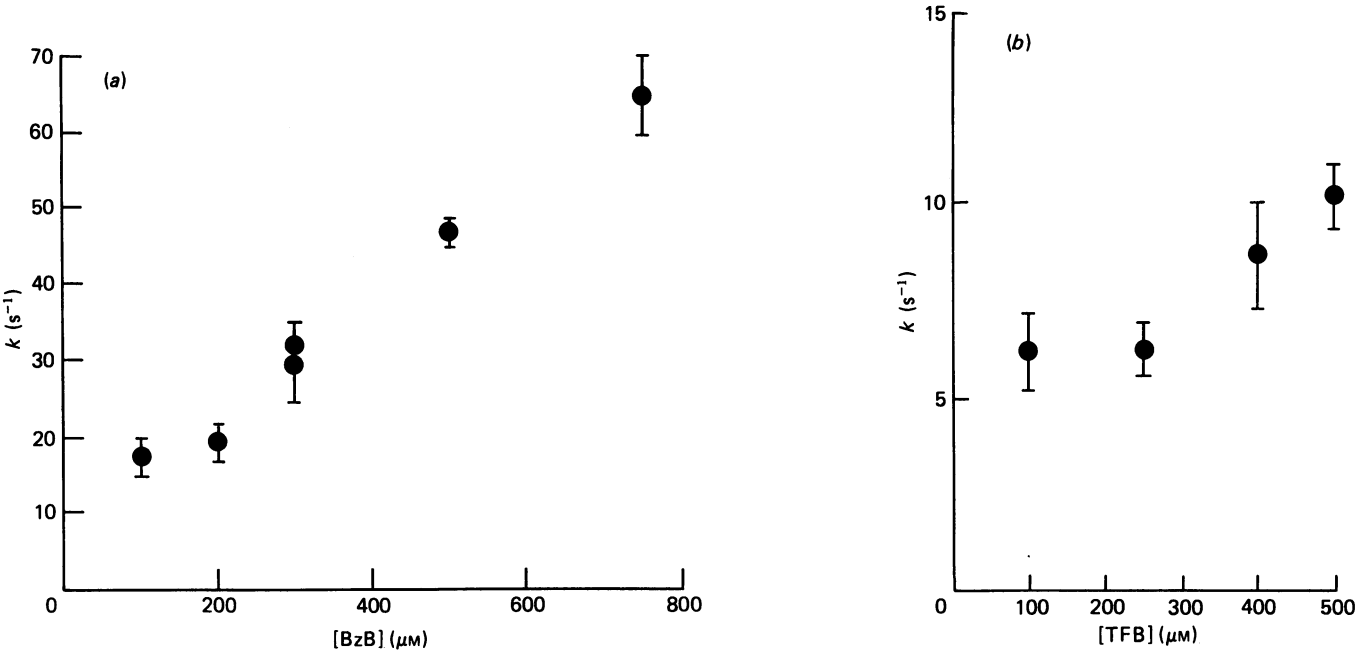


Fig. 3. Variation of rate constant for transition to steady state with concentration of inhibitor

The reactions were carried out in the stopped-flow spectrophotometer at 25 °C. (*a*) β-Lactamase I and BzB (I). (*b*) β-Lactamase I and TFB (II).

The variation of k with concentration of inhibitor (Fig. 3) points to a two-step mechanism of slow-binding inhibition (Morrison, 1982; Williams & Morrison, 1979; Sculley & Morrison, 1986), in which a rapid equilibrium precedes a rate-determining step:

$$E + I \underset{k_{-2}}{\overset{K_1 \quad k_{+2}}{\rightleftharpoons}} EI \rightleftharpoons EI^*$$

The positive slope in Fig. 3 excludes the mechanism in which a slow conformational change of the enzyme precedes rapid binding of the inhibitor (Duggleby *et al.*, 1982). Had these plots been clearly curved, the mechanism consisting of slow one-step binding would have been excluded. In practice, it was not possible to do experiments at high enough concentrations of inhibitor to distinguish between a straight line and a hyperbola.

However, one-step slow binding is not a chemically satisfactory mechanism for interaction of protein with a ligand: the encounter rate must be large. Additionally, v_0 showed dependence on i in accordance with eqn. (6). The overall dissociation constant, K_i^*, is then related to the dissociation constant of the first step, K_i, by:

$$K_i^* = K_i \cdot \frac{k_{-2}}{k_{+2}+k_{-2}} \quad (5)$$

We have used the kinetics program KINSIM (Barshop *et al.*, 1983) to explore the conditions favourable to seeing a burst of product. The burst, when the last added component was enzyme, was much more readily detected than was the lag, when the last component added was substrate (Fig. 4). To detect a lag would have required a higher concentration of substrate than was practicable. There is an optimum concentration of inhibitor to use, as discussed in the Appendix.

There are several methods of obtaining values for the parameters, but we preferred, as did Morrison & Cleland (1983), to obtain v_0, v_f and k by fitting progress curves to eqn. (3). The program of Duggleby (1984) was used for non-linear regression. Then v_0 and v_f were used to obtain K_i and K_i^* from eqns. (6) and (7):

$$v_0 = \frac{V_{max.}[S]}{[S]+K_m(1+i/K_i)} \quad (6)$$

$$v_f = \frac{V_{max.}[S]}{[S]+K_m(1+i/K_i^*)} \quad (7)$$

The concentration of inhibitor (i) was 20–100 times the concentration of enzyme, and so no corrections to the concentration of inhibitor needed to be made for the inhibitor binding to the enzyme. The concentration of substrate was regarded as constant, at its initial value, and conditions were arranged so that there was no more than 10% consumption of the substrate at the end of the transient. The rate constant k_{-2} was obtained from:

$$k_{-2} = \frac{v_f}{v_0} \cdot k \quad (8)$$

by plotting v_0/v_f against k, and then k_{+2} was obtained from:

$$k_{+2} = k_{-2}\left(\frac{K_i}{K_i^*}-1\right)$$

Also:

$$k = k_{-2}+k_{+2}\left(\frac{i/K_i}{1+[S]/K_m+i/K_i}\right)$$

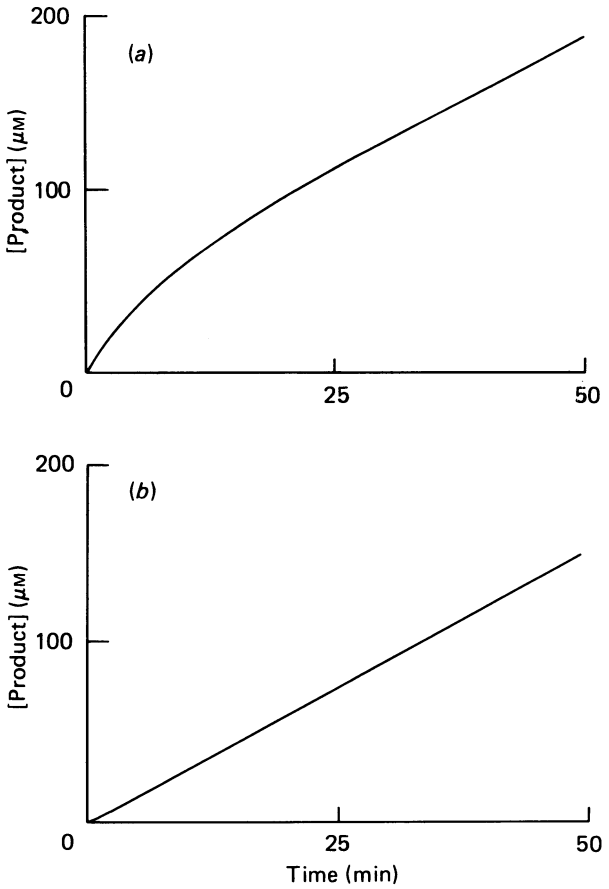


Fig. 4. Simulations for two-step binding

Stimulations were carried out with KINSIM program of Barshop *et al.* (1983) for enzyme species and product. The parameters were: $k_{cat.}$ 269 min^{-1}, K_m 1.5 mM, K_i, 1.46 μM, k_{+2} 0.57 min^{-1} and k_{-2} 0.05 min^{-1}. The concentrations of substrate, enzyme and inhibitor were 1000 μM, 0.1 μM and 0.5 μM respectively. (*a*) Enzyme added last; (*b*) substrate added last.

DISCUSSION

The results from the binding of three inhibitors to two β-lactamases are given in Table 1. The dissociation constants K_i and K_i^* tend to be lower at low temperatures, which may be due partly to the different conditions and partly to the change in enthalpy on binding. In aqueous media at ordinary temperatures the rate constants for decomposition of the complex EI* to EI, k_{-2}, did not vary greatly for the three systems, but k_{+2} varied more. The ratio k_{+2}/k_{-2} was in the range 2.9–25; this may characterize a change in enzyme conformation (see below).

The binding of BzB (I) to β-lactamase I may be compared with the binding of penicillin G, as reflected in the K_m, which may well be a dissociation constant (Waley, 1975). The K_i^* was 14.3 μM, and the K_m was 80 μM, but the K_i was 256 μM. Thus, when we compare the boronic acid with the substrate, the initial binding is weaker but the final binding is tighter. This is consistent with EI* mimicking the tetrahedral intermediate in the enzymic reaction.

The crystallographic results obtained by Herzberg & Moult (1987) on the homologous β-lactamase from *Staphylococcus aureus* PC1 suggest the interactions that would stabilize the putative tetrahedral complex. The main-chain nitrogen atoms of Ser-70 and Gln-237 would form hydrogen bonds with the boronate oxygen atoms; the positively charged NH_3^+ group of Lys-73 would interact with the negatively charged boronate and the proton could perhaps be taken up on Glu-166. Clearly, experimental evidence is required to test these ideas.

The change of EI to EI* could reflect the change from trigonal to tetrahedral boron, a change in enzyme conformation or both. It seems quite likely that there is

Table 1. Thermodynamic and kinetic parameters for the binding of boronic acids to β-lactamases

The enzymes were β-lactamase I from *B. cereus* (βI) or the β-lactamase from *Pseudomonas aeruginosa* (Ps), and the inhibitors were BzB, TFB and PhB. The solvent used at low temperatures was 50%-saturated ammonium acetate for enzyme βI at −30 °C and 40% (v/v) dimethyl sulphoxide in 100 mM-KCl/10 mM-sodium cacodylate buffer, pH* 8.1, at −24.5 °C for enzyme Ps. At ordinary temperature (25 °C) βI was used in 0.5 M-NaCl/20 mM-Mes buffer, pH 6, and enzyme Ps in 0.1 M-NaCl/20 mM-Mops buffer, pH 7.

Low temperature

Enzyme	Inhibitor	K_i (μM)	K_i^* (μM)	k_{+2} (min^{-1})	k_{-2} (min^{-1})	k_{+2}/k_{-2}
βI	BzB(I)	4.06±0.60	0.41±0.001	0.215±0.004	0.0242±0.0008	8.9
βI	TFB(II)	69±19	4.47±1.60	0.26±0.16	0.018±0.001	14
Ps	PhB	1.46±0.20	0.117±0.008	0.57±0.14	0.05±0.002	12

Ordinary temperature

Enzyme	Inhibitor	K_i (μM)	K_i^* (μM)	k_{+2} (s^{-1})	k_{-2} (s^{-1})	k_{+2}/k_{-2}
βI	BzB(I)	256±18	14.3±2.7	60.6±23.0	3.57±0.43	17
βI	TFB(II)	33.5±2.8	8.58±0.98	6.55±1.50	2.24±0.08	2.9
Ps	PhB	13.5±3.4	0.524±0.090	218±110	8.80±0.69	25

a change in conformation, and certainly the values of k_{+2} in Table 1 are lower than those reported by Nakatani *et al.* (1975) for the interconversion of trigonal and tetrahedral boron in the interaction of PhB and subtilisin. These changes are also slower than any changes in conformation during catalysis. The value of $k_{cat.}$ for penicillin G (Waley, 1975) places a limit of the order of 1 ms for the time taken for conformational changes during catalysis. This contrasts with times of several hundred milliseconds for the changes induced by the boronic acids. Thus the rate of protein conformational change associated with formation of the tetrahedral adduct (intermediate) appears to depend on the nature of the ligand in the initial complex.

Although TFB (II) binds more tightly than BzB (I) to β-lactamase I at 25 °C, the opposite is true in aqueous ammonium acetate at −30 °C. When PhB is compared with the substituted boronic acids studied previously (Beesley *et al.*, 1983) it is found that the unsubstituted compound binds about 5 times more tightly than the best compound in the earlier series.

The boronic acid structurally related to methicillin, MeB (III), did not give biphasic progress curves, and had a somewhat higher inhibition constant for β-lactamase I (96 μM at pH 7 at 30 °C) than BzB (I) to TFB (II). Methicillin is a substrate for β-lactamase I that is believed to cause a change in conformation of the acylated enzyme (Kiener & Waley, 1977), and it may be that there is a change in conformation when MeB binds that is effectively synchronous with binding and so is not detected as a separate step. One aspect of the interaction between methicillin and β-lactamase I is that the enzyme is rendered more susceptible to thermal denaturation (Citri, 1981), but we could only detect the opposite effect, namely a modest stabilization by the boronic acid MeB (III).

The results reported for the specific boronic acids (e.g. BzB, I) invite comparison with the peptide boronic acids that are slow-binding inhibitors of serine proteinases; the slow step is believed to be a change in conformation of the enzyme (Kettner & Shenvi, 1984). Some of these latter inhibitors bind very tightly, with K_i^* below 1 nM; none of our inhibitors binds to the β-lactamases with such a high affinity. Moreover, our inhibitors would not be classed as slow-binding inhibitors if the criteria of Imperiali & Abeles (1986) were used, the k_{off} (k_{-2}) values being considerably greater than 0.05 s^{-1}, and progressive inhibition not being observed under ordinary conditions of assay. It was only by extending the conditions that we were able to detect slow-binding inhibition, and thence to show a two-step mechanism. This suggests that more, perhaps many more, examples of enzyme inhibition might involve two-step binding. This, in turn, suggests that a general feature of recognition may be reorganization of the macromolecule after the initial encounter has taken place. The mechanism may be quite normal, but, often, hard to detect.

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APPENDIX

Conditions for a high burst number

The size of the burst in slow-binding inhibition may be defined in terms of the dimensionless burst number, B, as:

$$B = \frac{v_0 - v_f}{k \cdot e_0}$$

Use of eqn. (8) in the text then gives:

$$B = v_f[1 - (v_f/v_0)]/(k_{-2} \cdot e_0)$$

Then use of eqns. (6) and (7) in the text followed by partial differentiation leads to the condition that $(\partial B/\partial i) = 0$ is that:

$$i_{opt.} = K_i^*(1 + [S]/K_m)$$

Moreover, the largest burst number is then:

$$B_{opt.} = \frac{V_{max.}}{4k_{-2} \cdot e_0}\left(\frac{i(1/K_i^* - 1/K_i)}{1 + i/K_i}\right)$$

If $i \gg K_i$ and $K_i^* \ll K_i$ then:

$$B_{opt.} = k_{cat.}/4k_{-2}$$