(1-Aminoethyl)boronic Acid: A Novel Inhibitor for *Bacillus stearothermophilus* Alanine Racemase and *Salmonella typhimurium* D-Alanine:D-Alanine Ligase (ADP-Forming)†

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ABSTRACT: (1-Aminoethyl)boronic acid (Ala-B), an analogue of alanine in which a boronic acid group replaces the carboxyl group, has been synthesized and found to inhibit the first two enzymes, alanine racemase (from *Bacillus stearothermophilus*, EC 5.1.1.1) and D-alanine:D-alanine ligase (ADP-forming) (from *Salmonella typhimurium*, EC 6.3.2.4), of the D-alanine branch of bacterial peptidoglycan biosynthesis. In both cases, time-dependent, slow binding inhibition is observed due to the generation of long-lived, slowly dissociating complexes. Ala-B inhibits alanine racemase with a K_i of 20 mM and a k_{inact} of 0.15–0.35 min^{-1}. Time-dependent loss of activity is paralleled by conversion of the 420-nm chromophore of initial bound PLP aldimine to a 324-nm absorbing species. On dilution of Ala-B, racemase activity is regained with a $t_{1/2}$ of ca. 1 h. The D-Ala-D-Ala ligase also shows progressive inhibition by Ala-B provided ATP (but not AMP-PNP or AMP-PCP) is present. The presence of D-alanine along with ATP also leads to Ala-B-induced inactivation. Kinetic analysis suggests Ala-B can compete with D-alanine at either of the two D-alanine binding sites, and on inactivation with Ala-B, labeled D-alanine, and labeled ATP, the inactive enzyme has stoichiometric amounts of D-alanine, ADP, P_i, and Ala-B bound. The half-life of inactive enzyme complexes varied from ~2 h (without D-alanine) to 4.5 days (with D-alanine). No D-Ala-D-Ala-B dipeptide was detected. The ability of Ala-B to generate slow binding inhibition of these two cell wall biosynthetic enzymes may relate to the electrophilic nature of the boron atom in the $-B(OH)_2$ group and the consequent propensity to add H_2O and so generate a tetrahedral boronate anion at the active site.

The peptidoglycan is an essential component of the bacterial cell wall, providing a rigid structure to protect bacteria from osmotic pressure. In the initial stages of the biosynthesis of the key peptidoglycan precursor UDP-*N*-acetylmuramyl-L-Ala-D-Glu-*meso*-diaminopimelate-D-Ala-D-Ala, three enzymes constitute a D-alanine-generating and -utilizing pathway (Rogers et al., 1980). L-Alanine is racemized to give D-alanine by the pyridoxal 5′-phosphate (PLP)[1] containing enzyme alanine racemase (EC 5.1.1.1). The D-alanine thus produced is coupled in an ATP-dependent reaction with a second D-alanine to give the dipeptide D-alanyl-D-alanine by D-alanine:D-alanine ligase (ADP-forming) (D-Ala-D-Ala ligase, EC 6.3.2.4). A third enzyme, the D-Ala-D-Ala adding enzyme (EC 6.3.2.15), catalyzes the formation of the UDP-muramyl-pentapeptide by coupling the D-Ala-D-Ala unit to UDP-*N*-acetylmuramyl-L-Ala-D-Glu-*meso*-diaminopimelate in a similar ATP-dependent reaction. Polymerization of this precursor with *N*-acetyl-D-glucosamine results in a repeating polyglycan strand which is now able to cross-link with an adjacent strand to extend the peptidoglycan. Work in these laboratories has centered on the mechanism of inhibition of both alanine racemase (Badet et al., 1984; Roise et al., 1984; Copie et al., 1988) and D-Ala-D-Ala ligase (Duncan & Walsh, 1988) as potential targets for antibacterial design.

Boronic acids have been shown to be inhibitors of serine proteases (Ketner & Shenvi, 1984), aminopeptidases (Shenvi, 1986), and β-lactamases (Beesley et al., 1983; Crompton et al., 1988). The mechanism of inhibition for the proteases and β-lactamases appears to involve coordination of the active site nucleophilic serine hydroxyl on the boron atom while that for aminopeptidase inhibition is less clear. Matthews et al. (1975) have shown from crystallographic studies that the active site serine residue of subtilisin does indeed coordinate to boron, leading to a stable, tetrahedral boron geometry. Formation of such a tetrahedral borate anion at the active site (whether by serine hydroxyl or water attack), which acts as a transition-state analogue of the normal tetrahedral carbonyl addition adduct, is thought to explain the inhibition of these enzymes.

Herein, we report on the inactivation of the first two enzymes of the D-alanine branch of peptidoglycan assembly, the alanine racemase from the Gram-positive bacterium *Bacillus stearothermophilus* and the D-Ala-D-Ala ligase from the Gram-negative organism *Salmonella typhimurium*, with an α-amino boronic acid, Ala-B (Figure 1), an analogue of alanine with boronic acid replacing the alanine carboxylate. This class of compounds has previously been unknown as active site directed inhibitors of either PLP-containing enzymes or peptide

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[1] Abbreviations: PLP, pyridoxal, 5′-phosphate; PMP, pyridoxamine 5′-phosphate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PK-LDH, pyruvate kinase/lactate dehydrogenase; AMP-PNP, 5′-adenylyl imidodiphosphate; AMP-PCP, 5′-adenylyl methylenediphosphate; TLC, thin-layer chromatography; ATP, adenosine 5′-triphosphate.

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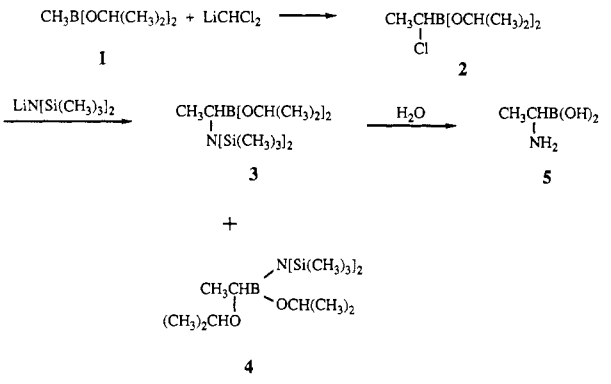
Alanine Ala-B

FIGURE 1: Comparison between alanine and (1-aminoethyl)boronic acid (Ala-B).

Scheme I: Synthesis of (1-Aminoethyl)boronic Acid (Ala-B)



bond forming enzymes and opens up another strategy for the design of inhibitors for these clinically important enzymes.

Experimental Procedures

Materials. L- and D-alanine, ATP, NADH, CHES, and HEPES were purchased from Sigma Chemical Co., St. Louis, MO. Lactate dehydrogenase (10 mg/mL; 550 units/mg), D-amino acid oxidase (5 mg/mL; 15 units/mg), pyruvate kinase/lactate dehydrogenase (4 mg/mL; 500 units/mg), 5′-adenylyl imidodiphosphate (AMP-PNP), and 5′-adenylyl methylenediphosphate (AMP-PCP) were from Boehringer-Mannheim Biochemicals, Indianapolis, IN. D-[^{14}C]Alanine, [^{14}C]ATP, and [γ-^{32}P]ATP were purchased from Amersham, Arlington Heights, IL. Centricon microconcentration filters (MW cutoff 30000) were purchased from Amicon, Lexington, MA.

D-Ala-D-Ala ligase was purified from *S. typhimurium* DB7000/pDS4 as described in Daub et al. (1988). The *B. stearothermophilus* alanine racemase was prepared according to the method of Neidhart et al. (1987). The enzymes were purified to >95% homogeneity as determined by polyacrylamide gel electrophoresis in the presence of SDS.

Synthesis of Diisopropyl [1-[Bis(trimethylsilyl)amino]-ethyl]boronate. (Dichloromethyl)lithium was prepared by adding 72 mL (0.115 mol) of 1.6 M *n*-butyllithium in hexane dropwise to a solution of 15 mL of dichloromethane in 175 mL of tetrahydrofuran stirred at −78 °C under argon (Matteson et al., 1983; Matteson & Majumdar, 1983). A solution of 16.6 g (0.115 mol) of diisopropyl methylboronate (**1**; Scheme I) (Brown & Cole, 1983) in 10 mL of diethyl ether was added dropwise to the cold stirred suspension, and the solid dissolved as the last of the boronic ester was added. The mixture was allowed to stand overnight and then heated to reflux for 1 h, which resulted in precipitation of lithium chloride, a positive indication of formation of (1-chloroethyl)boronic ester (**2**). This mixture was cooled to −78 °C and stirred under argon during the dropwise addition of a solution of lithiohexamethyldisilazane, which had been prepared by addition of 0.115 mol of butyllithium to 20.7 g (0.128 mol) of hexamethyldisilazane in 100 mL of tetrahydrofuran at −78 °C. The mixture was kept 3 h at 25 °C and then distilled. The material collected at 25–80 °C (0.01 Torr) was redistilled, and after very slow distillation, a major fraction was collected at 50–52 °C (0.02 Torr) and estimated by NMR to contain 73–76% of the major isomer **3** with the remainder being isomer **4**. Results from 200-mHz ^{1}H NMR ($CDCl_3$): isomer **3**, δ 0.12 (s, 12, $SiCH_3$), 1.13 [m, 15, $CH(CH_3)$ + $CHCH_3$], 2.73 (q, 1, $BCHCH_3$), 4.61 [septet, 2, $CH(CH_3)_2$]; isomer **4**, δ 0.16 (s, 12, $SiCH_3$), 1.13 [m, overlaps with 3, 15, $CH(CH_3)_2$ + $CHCH_3$], 3.37 (q, 1, $BCHCH_3$), 3.63 [septet, 1, $C-O-CH(CH_3)_2$], 4.83 [septet, 1, $B-O-CH(CH_3)_2$]. Mass spectrum: *m/e* calculated for $C_{14}H_{36}BNO_2Si_2$, 317.238; *m/e* found, 317.255 (± 0.034, five determinations). Addition of diisopropyl [1-[bis(trimethylsilyl)amino]ethyl]boronate (**3**) to water leads to immediate hydrolysis of the trimethylsilyl group and diisopropyl boronate to give racemic D,L-Ala-B (**5**). Ala-B is unstable in aqueous solution at 37 °C for long periods (>24 h); dilutions from stock were prepared fresh (in ethanol) and used the same day.

Analytical Methods. Racemase activity was measured routinely in 0.1 M HEPES buffer (pH 7.3) at 37 °C following the procedure of Badet et al. (1984), and D-Ala-D-Ala ligase activity was measured in continuous spectrophotometric assays (37 °C) of ADP formation utilizing PK-LDH, which has been described (Daub et al., 1988). Absorption spectra and activity readings were measured with a Hewlett-Packard diode array spectrophotometer. Gel filtration was carried out with a rapid desalting column (Pharmacia, Piscataway, NJ) attached to a Pharmacia FPLC apparatus; separation of enzyme from small molecules was achieved in less than 2 min.

Inactivation Kinetics. The time-dependent inactivation of alanine racemase by Ala-B was measured at 25 °C in 0.1 M HEPES, pH 7.3, with Ala-B concentrations ranging from 10 to 38 mM and racemase at 0.065 μM. At various time intervals, aliquots were taken, and activity was measured. Competitive inhibition was also measured by incubation of Ala-B (ranging in concentration from 0 to 23 mM) in the assay cuvette (0.1 M HEPES at pH 7.3) and addition of racemase at various substrate (L-alanine) concentrations. Measurement of steady-state parameters at different Ala-B concentrations allows calculation of competitive inhibition. Slow binding inhibition (Morrison & Walsh, 1988) was also measured by incubation of Ala-B (ranging in concentration from 5.8 to 23 mM) in an assay cuvette (0.1 M HEPES at pH 7.3) containing 10 mM L-alanine ($2K_m$) in a total volume of 1.0 mL. Inactivation of D-Ala-D-Ala ligase with Ala-B was measured by adding enzyme (88 pmol) to PK-LDH coupled assay mixtures containing ATP (5 mM), D-alanine (1–20 mM), and Ala-B (5–100 μM). Measurement of the initial rate and the rate of inactivation in the presence of 20 mM D-alanine was ascertained. In a second series of experiments, D-Ala-D-Ala ligase (88 pmol) was preincubated with ATP (5 mM) and Ala-B (500 μM) in a final volume of 20 μL before addition to cuvettes containing a range of Ala-B concentrations. Again the rate of inactivation in the presence of 20 mM D-alanine was ascertained. In the calculation of the Ala-B concentration for inactivation of the D-Ala-D-Ala ligase, only the D-Ala-B concentration was used (obtained by dividing the total concentration by 2), as this enzyme shows exclusive specificity for only the D isomer of amino acids and amino acid analogues.

Spectral Observations. A 1.0-mL solution containing alanine racemase (50 μM) was assayed for activity, and an absorption spectrum was obtained. Ala-B (total concentration of 4 mM) was added, and both activity and the absorption spectrum were monitored as a function of time. Thus, any effect that inactivation may have on the PLP–enzyme chromophore will be ascertained.

The effect of Ala-B and biphenylboronic acid on the PLP chromophore, free in solution, was also investigated. To a pyridoxal 5′-phosphate solution (ranging in concentration from 75 to 375 μM) in 0.1 M HEPES buffer at pH 7.3 was added Ala-B (230 mM) or biphenylboronic acid (4 mM). The solution was mixed and left at room temperature for 30 min. At this point, the absorbance at 388 nm, corresponding to PLP, was measured. Also, aliquots of the PLP–Ala-B mixture were diluted to ensure reversible formation of any complex.

Involvement of ATP and Substrate in the Inactivation of D-*Ala*-D-*Ala Ligase.* Incubation mixtures were prepared containing D-Ala-D-Ala ligase (88 pmol), Ala-B (500 μM), and ATP (5 mM) or containing ligase (88 pmol), Ala-B (500 μM), ATP (5 mM), and D-alanine (1 or 20 mM) in a final volume of 20 μL. After 30 min at 37 °C, phosphoenolpyruvate, NADH, and PK-LDH were added, and incubation was continued for 5 min. This step was required to remove any ADP formed in the incubation mixture which would interfere with the initial rate of the subsequent assay. The final mixture was added to a 1-mL assay cuvette containing 20 mM D-alanine, and the rate of ADP release was monitored. Dilution effectively removed the inhibitor from the enzyme, leaving the concentration of Ala-B in the assay at 10 μM. Similar incubations were set up with AMP-PNP and AMP-PCP in place of ATP, and the enzyme activity was measured after dilution.

The P_i content of the enzyme·Ala-B·ATP complex was measured by the malachite green assay (Lanzetta et al., 1979). D-Ala-D-Ala ligase (16 nmol) was incubated at 37 °C with Ala-B (5 mM) and ATP (5 mM) (final volume 200 μL). The P_i concentration was determined at 5, 10, 20, and 30 min after enzyme addition and compared with P_i release from a similar incubation without enzyme.

Inactivation of D-*Ala*-D-*Ala Ligase with Labeled ATP and* D-*Alanine.* A total of 5 nmol of D-Ala-D-Ala ligase was inactivated as described above in the presence of labeled D-alanine or ATP. The following incubation mixtures were used: (A) Ala-B (5 mM), [γ-^{32}P]ATP (1 mM, 10 nCi nmol^{-1}); (B) Ala-B (5 mM), ATP (1 nM), D-[^{14}C]alanine (20 mM, 1 nCi nmol^{-1}); (C) Ala-B (5 mM), [^{14}C]ATP (1 mM, 10 nCi nmol^{-1}), D-alanine (20 mM); (D) Ala-B (5 mM), [γ-^{32}P]ATP (1 mM, 10 nCi nmol^{-1}), D-alanine (20 mM). Unbound label and excess inhibitor were removed by gel filtration, and radiolabeled samples were prepared for thin-layer chromatography (TLC) by the following procedure. Immediately after emergence from the column, the concentration of enzyme-bound label was increased by centrifugation through a Centricon filtration unit (30-kDa cutoff limit), thereby reducing the volume from 1 mL to approximately 50 μL. The concentrated material was heated (100 °C, 2 min), chilled on ice (15 min), and centrifuged to remove denatured, precipitated protein. Samples labeled with D-[^{14}C]alanine were analyzed on Avicel F TLC as described in Daub et al. (1988); [^{14}C]ATP and [γ-^{32}P]ATP were analyzed on PEI–cellulose TLC as previously described (Duncan & Walsh, 1988). The stoichiometry of labeling was determined by scintillation counting of an aliquot before concentration of the sample and was correlated with the enzyme specific activity measured within 2 min of emergence from the column.

Return of Activity. To determine if the inactivation of alanine racemase by Ala-B is reversible, activity was assayed upon dilution of the enzyme–inhibitor complex well below the apparent K_i. A total of 1.0 mL of an alanine racemase solution (65 μM) was inactivated with Ala-B (4 mM). A 1-μL aliquot was diluted into 1 mL of buffer and activity measured as a function of time by dilution into a standard D-alanine assay mixture (Badet et al., 1984). The remaining inactive enzyme solution (containing 65 μM enzyme) was placed on a Sephadex G-25 (40 mesh) sizing column (1.0 × 13 cm) at 4 °C and washed with buffer, thus separating enzyme from excess inhibitor. Activity was then measured as a function of time at 25 °C.

Incubation mixtures were prepared containing D-Ala-D-Ala ligase (5 nmol), Ala-B (5 mM), and ATP (5 mM) or containing ligase (5 nmol), Ala-B (5 mM), ATP (5 mM), and D-alanine (1 or 20 mM) in 25 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$ (final volume 200 μL). Inactivation was carried out by incubation at 37 °C for 1 h. Excess inhibitor, ATP, and substrate were removed by gel filtration, and enzyme-containing fractions were pooled and stored on ice. Aliquots were removed and assayed for activity at intervals over a number of hours or days.

Results

Synthesis of Ala-B. Ala-B, the boronic acid analogue of the amino acid D,L-alanine, was synthesized as detailed in Scheme I. Reaction of lithiohexamethyldisilazane with the (chloroethyl)boronic ester gave the N,O-protected Ala-B (**3**) plus the minor isomer **4**. Addition of water led to immediate hydrolysis of the silyl and isopropyl groups to yield Ala-B and the minor isomer in solution. Isomer **4** is not a structural analogue of an amino acid and should not be an obvious alanine analogue, but it was not removed from Ala-B solutions. Ala-B solutions are stable for a few hours at 4 °C in the indicated buffer systems. The decomposition pathway has not yet been analyzed.

Inactivation of Alanine Racemase by Ala-B. Incubation of the alanine racemase from *B. stearothermophilus* with Ala-B leads to time-dependent inhibition of the enzyme. A plot of $1/k_{obsd}$ vs $1/[\text{Ala-B}]$ gives a straight line in which the values for K_i and k_{inact} are 20 ± 1 mM and 0.35 ± 0.02 min^{-1}, respectively. This is shown in Figure 2 (inset). Addition of enzyme to an Ala-B mixture containing saturating L-alanine leads to virtually no loss of activity, indicating substrate protection. Thus, Ala-B can be identified as a time-dependent, active site directed inhibitor. Experiments designed to look for competitive inhibition revealed reversible binding between Ala-B and enzyme. Slow binding inhibition, as described for the D-Ala-D-Ala ligase (vide infra), was also observed. The value for k_{inact} (0.15 min^{-1}) obtained via this method thus compares reasonably to the value obtained by time-dependent inhibition experiments.

From the information obtained by the kinetic data, one can present an inactivation scheme, shown in eq 1. Incubation

$$E + I \rightleftarrows EI \rightleftarrows EI' \rightleftarrows EI'' \quad (1)$$

of Ala-B (I) with racemase (E) leads to formation of a reversible complex (EI) with a binding constant of >50 mM. The value for K_i (20 mM) obtained by time-dependent inhibition studies suggests that this binding constant represents a composite of equilibria. Thus, a three-step inactivation mechanism can describe the kinetic data.

Although the data allow a schematic representation of the kinetic inactivation mechanism, they provide little information as to any structural changes which occur. Incubation of racemase with Ala-B leads to a change in the absorbance spectrum of the PLP–enzyme chromophore which is identical with the rate of loss of activity. This is seen in Figure 2. The absorbance at 420 nm, characteristic of a protonated Schiff base, is shifted to 324 nm, characteristic of PMP derivatives. Separation of the enzyme fraction by column chromatography

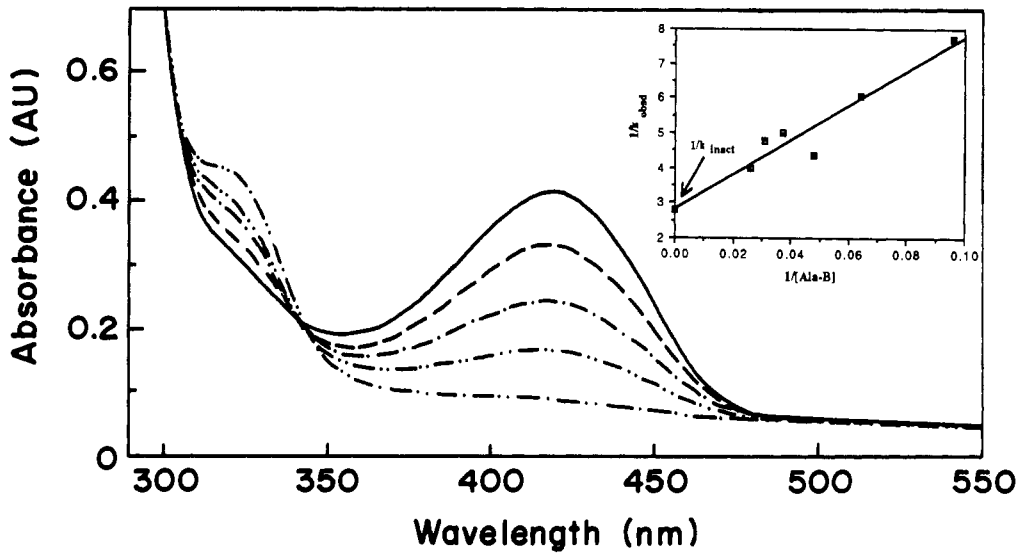


FIGURE 2: Visible spectrum of alanine racemase after reaction with Ala-B. Alanine racemase (28 μM) in buffer (0.1 M HEPES, pH 7.3); alanine racemase incubated with Ala-B at 100% activity (—), 73% activity (– –); 47% activity (–·–), 30% activity (–··–), and 2% activity (–···–). Inset: Inactivation of the *B. stearothermophilus* alanine racemase with Ala-B. Measurement of the inactivation rate at various Ala-B concentrations allows calculation of K_i and k_{inact}.

shows unequivocally that the 324-nm chromophore is enzyme-associated. Model studies show that incubation of PLP (in the presence and absence of alanine) with Ala-B leads to an immediate loss of the absorbance at 388 nm (λ_{max} for PLP at pH 7.3) and the presence of a new peak (λ_{max} = 300 nm). Apparently, Ala-B interacts with PLP such that it quenches the long-wavelength chromophore. In order to determine whether this was exclusive for amino boronic acids, biphenylboronic acid was used in a control experiment. Addition of biphenylboronic acid to a PLP solution (in the presence and absence of alanine) led to a loss in the absorbance at 388 nm and the appearance of a peak at 306 nm. As biphenylboronic acid contains no amino group which can form a Schiff base with PLP, the change in the absorbance spectra is believed to be due to chelation of the boronic acid on the PLP hydroxyl group and aldehydic oxygen (or to the amino group from the alanine–PLP Schiff base). However, addition of biphenylboronic acid to active enzyme led to no change in the spectrum, nor did it lead to any inhibition; thus, Ala-B must inhibit the enzyme by a distinct mechanism, and one that is active site directed.

Regain of Racemase Catalytic Activity from Inhibited Enzyme Complex. As noted, inactivation of alanine racemase by Ala-B occurs in a time-dependent manner to yield an inactive enzyme complex. Dilution of the complex such that the inhibitor is substantially below K_i leads to a slow return in the enzymatic activity. The rate of return, k_{rgn}, was 0.01 ± 0.005 min^{-1} ($t_{1/2} \sim 70$ min), with ca. 60–70% of total initial enzyme activity returned. To determine whether the lower activity is due to apoenzyme formation, pyridoxal 5′-phosphate was added to incubation mixtures but led to no further increase in total enzyme activity recovered.

Inhibition of D-*Ala*-D-*Ala Ligase by Ala-B.* Addition of D-Ala-D-Ala ligase, the second enzyme in the D-alanine branch of peptidoglycan biosynthesis, to assay mixtures containing Ala-B revealed inhibition of the rate of D-alanine processing (to produce D-Ala-D-Ala). The initial rate of the reaction was observed over a range of D-alanine and Ala-B concentrations and analyzed by the method of Dixon (1953) and Cornish-Bowden (1974). A plot of $1/v$ vs [I], shown in Figure 3a, is indicative of mixed inhibition in which the inhibitor interacts with enzyme alone and with the enzyme–substrate complex. Extrapolation of each line to the point of intersection allows

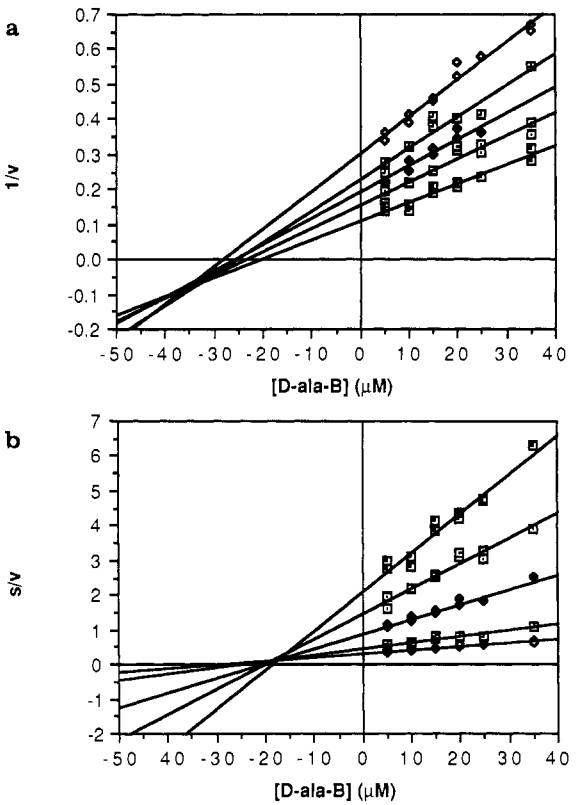


FIGURE 3: Inhibition of D-Ala-D-Ala ligase by Ala-B. (a) Plot of $1/v$ (arbitrary units) vs [I]. (b) Plot of s/v (arbitrary units) vs [I]. In each case, initial rates were measured for a range of D-alanine concentrations: 20, 10, 5, 2 and 1 mM.

one to obtain K_i, which is defined as the interaction of inhibitor with enzyme alone. A plot of s/v vs [I] (Figure 3b) allows one to arrive at K_i', which is the interaction of inhibitor with the ES complex. The values for K_i (for E + I ⇄ EI) and K_i' (for ES + I ⇄ EIS) are 35 μM and 18 μM, respectively.

From the shape of progress curves obtained in the presence of saturating D-alanine (20 mM) and over a range of Ala-B concentrations (data not shown), it was apparent that Ala-B was behaving as a time-dependent, slow binding inhibitor (Morrison & Walsh, 1988; Duncan & Walsh, 1988) for D-Ala-D-Ala ligase. Measurement of the reaction velocity at

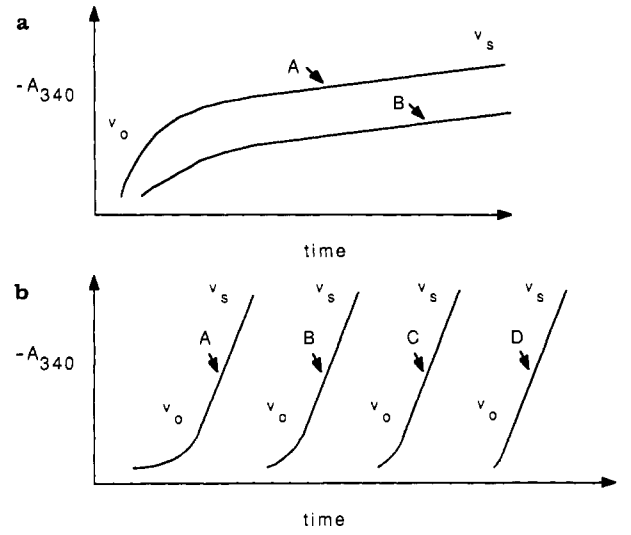


FIGURE 4: Illustration of the shape of D-Ala-D-Ala ligase progress curves. (a) Effect of preincubation of enzyme with Ala-B and ATP prior to assay: curve A is that of an assay in which the enzyme was added directly to a cuvette containing Ala-B; curve B was obtained after preincubation of D-Ala ligase with ATP and Ala-B before being added to the cuvette. The same final concentrations of D-alanine, ATP, and Ala-B were present in each case. (b) Regain of D-Ala-D-Ala ligase catalytic activity after inactivation in the presence of Ala-B, ATP, and 20 mM D-alanine. Curves A–D were obtained at approximately 2-h intervals. The initial rate, v_0, increases rapidly ($t_{1/2}$ = 2.4 h), but v_s increases very slowly ($t_{1/2}$ = 4.5 days).

various times during the onset of slow inhibition allows one to calculate the rate of approach to the steady state (k_{obsd}). The rate of formation of the initial EI complex (k_{on}) was calculated by averaging values of $k_{obsd}/[I]$ over the range of [I] used in this experiment and found to be 3200 M^{-1} s^{-1}. In order to determine the rate constant for the onset of slow inhibition (k_{inact}), $1/k_{obsd}$ was plotted against $1/[I]$ and, from the intersect of a line plotted through the points with abscissa, yielded k_{inact} = 20 min^{-1}.

The k_{obsd} was also calculated for a series of curves obtained after preincubation of enzyme with Ala-B and ATP (30 min at 37 °C; no substrate present). This was then diluted into an assay cuvette containing D-alanine, ATP, coupling enzymes, and requisite concentration of Ala-B. These data yielded k'_{inact} of 2.47 min^{-1}, and k'_{on} was 914 M^{-1} s^{-1}.

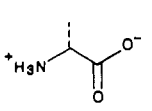
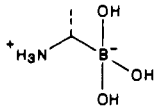
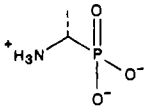
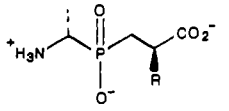
Requirement for ATP in the D-*Ala*-D-*Ala Ligase Slow Binding Inhibition Process.* It has previously been shown that the interaction of the slow binding inhibitory (aminoalkyl)phosphinate, D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid, with D-Ala-D-Ala ligase is ATP-dependent (Duncan & Walsh, 1988). When D-Ala-D-Ala ligase was incubated with Ala-B, and subsequently added to an assay mixture, no effect of the incubation was observed. The progress curve of the reaction was identical with that of an assay in which the same amount of enzyme was added directly to a cuvette containing the same final concentration of Ala-B. A similar incubation was carried out in the presence of ATP, and although the same steady-state rate was achieved on subsequent assay, the shape of the progress curve was different (Figure 4a), implying a different rate of approach to the steady state and thus a different rate of onset of slow inhibition. Therefore, the presence of ATP is essential for the inhibition process. Thus, slow binding inhibition takes place at both sites of interaction of enzyme with inhibitor, i.e., during formation of the E·I complex (actually E·I·ATP) and during formation of an E·S·I complex (actually E·S·ATP·I). In order to determine if ATP hydrolysis is necessary for formation of the initial EI complex, incubations were carried out with the two noncleavable ATP analogues, AMP-PNP and AMP-PCP. In both cases, no inhibition was detectable, and thus AMP-PNP and AMP-PCP cannot substitute for ATP. A possible explanation is that ATP hydrolysis is necessary for formation of the inhibited complex or because of structural hinderance due to the different conformation of the terminal phosphate in these analogues. This observation differs from that obtained upon incubation of D-Ala-P or (aminoalkyl)phosphinate with D-Ala-D-Ala ligase (Duncan & Walsh, 1988) as AMP-PNP can substitute for ATP with both of those inhibitors (K. Duncan and C. T. Walsh, unpublished results).

When D-Ala-D-Ala ligase was incubated with ATP and Ala-B, and P_i release was monitored over 30 min, it was found that there was an initial burst of P_i (after ca. 5 min) followed by a steady increase in P_i level. The initial release was approximately 1 equiv (with respect to enzyme concentration), and further P_i production (from apparent ATP hydrolysis) was at the rate of 3.5×10^{-2} equiv min^{-1}. This result suggests that there is a slow turnover process (one every 28.5 min) at this Ala-B binding site.

Regain of D-*Ala*-D-*Ala Ligase Catalytic Activity from Inhibited Enzyme Complexes.* In order to assess the rate of regain of catalytic activity from inhibited enzyme complexes, D-Ala-D-Ala ligase was inactivated, excess Ala-B inhibitor and ATP were removed by gel filtration, and the rate of return of activity was followed by measuring specific activity in aliquots over a period of time. The rate of regain was dependent upon the conditions under which the enzyme was inactivated. D-Ala-D-Ala ligase incubated with Ala-B and ATP had approximately 40% of full activity immediately after gel filtration. Activity was measured at intervals over an 8-h period, and from the specific activity at each time point, k_{rgn} was found to be 6.4×10^{-3} min^{-1} (k_{rgn}[a]). When D-Ala-D-Ala ligase was incubated with Ala-B, ATP, and D-alanine, different results were obtained at low D-alanine (1 mM) and high D-alanine (20 mM) concentrations. With 1 mM D-alanine in the preincubation, the D-Ala-D-Ala ligase activity immediately after the column step was found to be approximately 45% of full activity. The rate constant for return of activity (k_{rgn}[b]) was found to be 4.5×10^{-3} min^{-1}. The shape of the progress curves obtained at each point when the incubation was carried out in the presence of 20 mM D-alanine differed from those obtained with the 1 mM incubation. Upon gel filtration, there was initially very little (<1%) enzyme activity, but over a period of a few minutes in the assay cuvette, there was an increase in activity to a steady-state rate equivalent to 12% full activity. This rapid increase in activity must be due to rapid dissociation of an inactive complex because of the increased temperature in the normal assay (37 °C), whereas the pooled material was stored on ice. Following the increase in the initial rate of reaction, measurement of activity as a function of time led to calculation of the return of activity (k_{rgn}[c]) which is 4.8×10^{-3} min^{-1}. Over the same time interval, the final steady-state rate increased very slowly (Figure 4b). The increase in this rate was monitored over 4 days until it had increased to approximately 50% of full activity. From the rate of this increase, k_{rgn}[d] was calculated to be 1.1×10^{-4} min^{-1}.

Structure of the Inhibited Enzyme Complex. In order to ascertain the composition of the inhibited enzyme complex, radiolabeled substrate and ATP were utilized, as no labeled Ala-B was available. Incubation of ligase with Ala-B and [γ-^{32}P]ATP (incubation A) led to formation of an enzyme complex that after gel filtration gave ~0.9 mol of radiolabel/mol of inactive enzyme. Since D-Ala-D-Ala ligase does

Table I: Dissociation Rates of Various Inhibitors with the Alanine Racemase from *B. stearothermophilus* and the D-Ala-D-Ala Ligase from *S. typhimurium*

enzyme	 D-alanine	 Ala-B	 Ala-P	 (aminoalkyl)phosphinate
alanine racemase	substrate	slow binding inhibitor ($t_{1/2} \sim 1$ h)	slow binding inhibitor ($t_{1/2} \sim 25$ days)	no effect
D-Ala-D-Ala ligase	substrate	slow binding inhibitor ($t_{1/2} \sim 2$ h)	slow binding inhibitor ($t_{1/2} \sim 2$ min)	slow binding inhibitor ($t_{1/2} \sim 8$ h)

not bind ATP in the absence of substrate (Duncan & Walsh, 1988), this inactive complex must consist of enzyme·Ala-B·ATP (or fragments thereof). When D-alanine was included in the incubation, ~0.7 equiv of [^{14}C]-D-alanine (incubation B), ~1.0 equiv of [^{14}C]ATP (incubation C), and ~0.9 equiv of [γ-^{32}P]ATP (incubation D) were bound. TLC analysis revealed that in incubation A the label was present in P_i, not in ATP. This is convincing evidence that binding of Ala-B, concomitant with ATP binding, immediately results in ATP hydrolysis in which both ADP and P_i are bound at the active site. In incubation B, the label was in D-alanine; no spot corresponding to D-alanyl-D-alanine or to a dipeptide containing Ala-B was observed. Analysis of incubation C and incubation D revealed label in ADP and P_i, respectively; in neither case was a spot corresponding to ATP observed.

Discussion

(1-Aminomethyl)boronic acid (Ala-B) is a powerful slow binding, time-dependent inhibitor of both the alanine racemase from *B. stearothermophilus* and the D-Ala-D-Ala ligase from *S. typhimurium*. Although boronic esters are known to be inhibitors of serine proteases and active site serine β-lactamases, very little has been reported in investigating the inhibitory properties of these compounds to other classes of enzymes. The inactivation of a PLP-containing racemase as well as a peptide bond forming, ATP-cleaving ligase by an α-amino boronic acid suggests that other mechanistic enzyme types may indeed be susceptible to inhibition.

Alanine Racemase. From the initial data accumulated, the mechanism of inactivation of the Gram-positive *B. stearothermophilus* alanine racemase by Ala-B is hypothesized to proceed via eq 1. The unusual feature of this mechanism is loss of the 420-nm chromophore, suggesting that the inactive complex is not a protonated Schiff base. A possible explanation for the change in the absorption spectra upon inactivation may be that on formation of the enzyme–PLP–Ala B Schiff base, intramolecular addition of the boron hydroxyl ligand on the pyridoxal aldimine electrophilic carbon occurs to yield a cyclic pyridoxal carbinolamine derivative. It has been shown that cysteine and pyridoxal form an analogous five membered ring structure, in which nucleophilic attack of sulfur on the pyridoxal 4′-carbon leads to a thiazolidine ring (Abbott & Martell, 1970). An alternate mode of cyclization involving attack of the PLP phenolate oxygen on the $B(OH)_2$ moiety of an Ala–B–PLP aldimine is also possible, though that would give a seven-membered ring incorporating a tetrahedral borate. Model studies involving Ala-B and PLP were inconclusive to date however, as loss of the PLP chromophore occurs with Ala-B and biphenylboronic acid, which suggests that coordination of PLP to boron, e.g., via the phenolic OH to boron, can lead to a chromophore shift. It is unlikely that this (coordination of PLP hydroxyl group to boron) is the reason for loss of activity as racemase inactivation is specific for only Ala-B while other boronic acid analogues led to no appreciable loss in activity and is preventable by substrate binding.

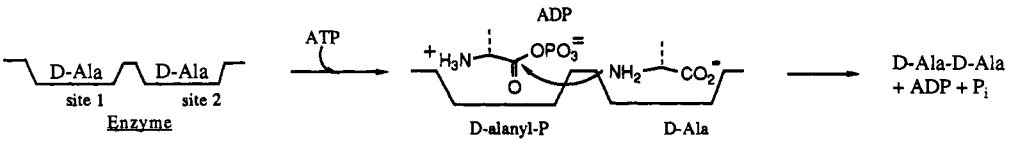
The inactivation of the *B. stearothermophilus* alanine racemase by Ala-B appears to present a novel mechanism for inhibition. As with β-fluoro- and β-chloroalanine inactivation (Badet et al., 1984; Roise et al., 1984), it operates by tying up the PLP cofactor. However, unlike these and other β-substituted alanine inhibitors (Badet et al., 1986; Faraci & Walsh, 1989), Ala-B incubation leads to only transient inhibition, as activity is restored upon dilution of the enzyme–Ala-B mixture ($t_{1/2} \sim 60$–70 min). The return of activity rate is roughly 10-fold less than the inactivation rate; thus, the inactive complex can be isolated and observed. We have previously reported that the corresponding phosphonate analogue of alanine, Ala-P (D or L), is also a time-dependent inactivator of the *B. stearothermophilus* alanine racemase by a slow binding, slow release process (Badet et al., 1986; Copie et al., 1988), but the off rate in that instance is extremely slow ($t_{1/2} \sim 25$ days).

D-Ala-D-Ala Ligase. Inhibition of the D-Ala-D-Ala ligase by Ala-B does not appear to follow the pattern of simple competitive kinetic behavior which was observed for the inhibitors D-cycloserine, D-Ala-P, (1-aminoethyl)phosphinate, and the (aminoalkyl)phosphinate, D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid (Parsons et al., 1988; Duncan & Walsh, 1988). The kinetic pattern of inhibition by Ala-B suggests mixed inhibition, i.e., more than one significant mode of interaction between enzyme and inhibitor. The K_i and K_i' values of 35 μM and 18 μM can be compared with K_i values of 15 μM for D-cycloserine (K. Duncan and C. T. Walsh, unpublished results), 500 μM for D-Ala-P, 400 μM for (1-aminoethyl)phosphinate, and 1.2 μM for the (aminoalkyl)-phosphinate (Duncan & Walsh, 1988). Although individual K_m values have not yet been determined for each D-alanine binding site on *S. typhimurium* D-Ala-D-Ala ligase, a valid comparison of the Ala-B inhibition constant values can be made with K_m values of 660 μM and 10 mM for the first and second D-alanine binding sites, respectively, for *Streptococcus faecalis* D-Ala-D-Ala ligase (Neuhaus, 1962). Thus, Ala-B has a much stronger binding affinity for each site than D-alanine. Compared to a K_i of 20 mM for alanine racemase (above), Ala-B is at least 600-fold more potent as an inhibitor of D-Ala-D-Ala ligase than alanine racemase.

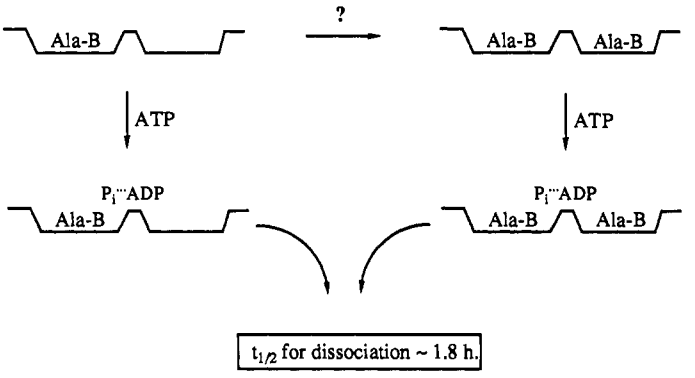
As observed for the (aminoalkyl)phosphinate inhibitor and D-Ala-P, Ala-B displays slow binding inhibition, as noted in Table I [see also Duncan and Walsh (1988)]. However, in this case, the behavior is more complicated in that we not only observed different K_i and K_i' values, but we also observed two different values for the rate of inactivation (k_{inact} and k'_{inact}), depending on preincubation conditions. In order to explain these phenomena, we must consider the proposed mechanism for D-Ala-D-Ala ligase. The normal reaction is thought to proceed in two steps, the first of which is binding of a substrate D-alanine molecule together with ATP, followed by enzyme-catalyzed ATP cleavage yielding an enzyme-bound acyl phosphate, ADP intermediate. In the second step, the acyl phosphate suffers nucleophilic attack by the amino nitrogen

Scheme II: Proposed Mechanism of D-Ala-D-Ala Ligase Catalysis and Inhibition

case 1. Normal turnover

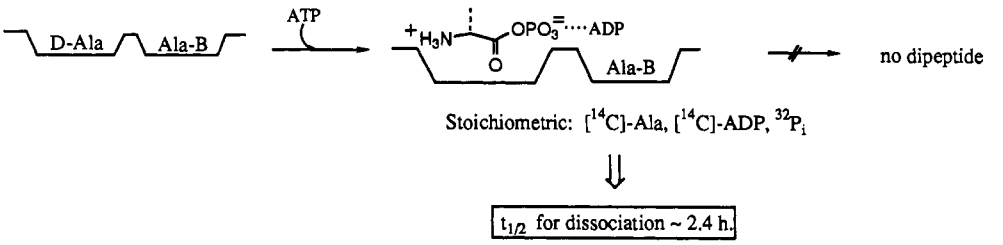


case 2. Inhibition: Ala-B + ATP

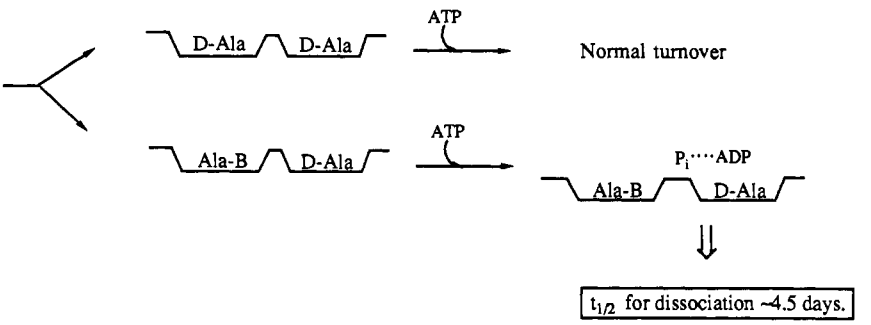


case 3. Inhibition: Ala-B + D-Ala + ATP

(a) low [D-Ala]



(b) high [D-Ala]



of the second substrate D-alanine molecule, displacing the phosphate moiety and releasing product D-Ala-D-Ala (case 1, Scheme II). In accord with the kinetic data of Figure 3, we reason that Ala-B is able to effectively compete with D-alanine at both D-alanine binding sites (N-terminal and C-terminal for dipeptide assembly) and that the two values for k_{inact} suggest time-dependent inhibition occurring at both interaction sites. k_{inact} represents the overall rate of inactivation and is a composite of the separate inactivation rates at the first and second Ala-B binding sites. This is due to the high concentration of D-alanine present in the assay, which effectively saturates both D-alanine binding sites. k'_{inact}, however, was determined after prior saturation of the first binding site with Ala-B and thus is a reflection of the rate of inactivation at the second D-alanine binding site. The value obtained, 2.47 min^{-1}, was approximately 8-fold lower than the overall rate (20 min^{-1}) (aggregate of two processes). The values obtained for k_{on} and k'_{on}, 3200 and 914 M^{-1} s^{-1}, respectively, are several orders of magnitude lower than typical bimolecular rate constants [10^5–10^7 M^{-1} s^{-1} for ES formation (Fersht, 1984)], which suggests that more than one step occurs in formation of the first detectable EI complex in each case (Morrison & Walsh, 1988).

An attempt to measure the rate of regain of catalytic activity from fully inhibited D-Ala-D-Ala ligase revealed that this rate was dependent on a number of factors, including both the presence of and concentration of D-alanine in the preincubation enzyme inactivation assay mix. When D-Ala-D-Ala ligase was incubated with Ala-B alone, no long-term inhibition was observed. However, when ATP was included in the incubation mix, inactive enzyme was found to regain activity (k_{rgn}[a]) with a half-life of approximately 1.8 h [although substantial

enzyme activity (>40%) was found immediately off the column]. Thus, either Ala-B must be denied access to the D-Ala-D-Ala ligase active site, or it does not form a stable, long-lived complex in the absence of ATP, as was found in the previous cases of time-dependent inhibition of this enzyme (Duncan & Walsh, 1988). Preincubation in the presence of a low D-alanine concentration again yielded a single release rate, $k_{rgn}[b]$, with a 2.5-h half-life. However, when high D-alanine concentrations were used, two rate constants for return of activity were measurable from enzyme which had very little activity after gel filtration. One of these, $k_{rgn}[c]$, had a half-life of 2.4 h, which we propose is the same rate measured at low D-alanine concentrations ($k_{rgn}[b]$), and accounts for ~12% of the total enzyme activity. The final regain rate, $k_{rgn}[d]$, is that of a very long lived complex (about 88% of the remaining enzyme activity) and has a half-life of around 4.5 days. Thus, it appears as if there is a number of different enzyme–substrate–inhibitor interactions which lead to these unusual kinetic observations. The ability of D-alanine to bind in two different sites [with different dissociation constants at each site (Neuhaus, 1962)] and of Ala-B to bind to each of these sites independently or together is most likely the primary reason. The binding of radiolabeled ATP to inactivated enzyme shows that in each case inactive enzyme has ATP bound in a 1:1 stoichiometry.

These regain rates can be compared (Table I) with the half-life for the enzyme·ATP·(aminoalkyl)phosphinate transition-state analogue which was found to be 8.2 h (Duncan & Walsh, 1988). While these release rates are dramatically slow, a number of time-dependent enzyme inhibitors have been studied which have exceptionally long half-lives (Morrison & Walsh, 1988). Among these is Ala-P (Table I), which has a $t_{1/2}$ for regain of 25 days with the *B. stearothermophilus* alanine racemase (Badet et al., 1986).

Incubations carried out with Ala-B in the presence of the radiolabeled D-alanine and ATP revealed that in each case ATP is quantitatively cleaved to ADP and P_i. Even in the absence of substrate D-alanine, no ATP was detected in the enzyme·Ala-B·nucleoside phosphate complex after gel filtration. Thus, either product fragments ADP and P_i are very tightly bound to the enzyme while Ala-B is bound, or possibly, a transient, bound phosphorylated Ala-B forms which hydrolyzes immediately upon enzyme denaturation. This would be analogous to the proposed acyl phosphate intermediate believed to be formed after binding the first D-alanine in the normal reaction mechanism. We have previously noted that D-Ala-P (Table I) transiently inhibits D-Ala-D-Ala ligase with ATP fragmentation, raising the possibility of a bound phosphorylated Ala-P species. A boronophosphate intermediate would be undetectable away from the enzyme active site as it would be susceptible to hydrolysis in aqueous solution (Gerrard, 1961), but it is questionable whether the B–OH hydroxyl group is a competent nucleophile for γ-PO_3^{2-} transfer from ATP. By monitoring the release of inorganic phosphate from an incubation of D-Ala-D-Ala ligase with Ala-B and ATP, a slow catalytic rate of ATP hydrolysis (once every 28 min) was observed, implying that there is some ATPase activity displayed by D-Ala-D-Ala ligase in the presence of Ala-B (slow release and then rapid breakdown of an Ala-B-PO_3^{2-}?). When radiolabeled D-alanine was added to ATP, Ala-B, and enzyme, stoichiometric association of alanine with the inactive enzyme complex was observed, but the absence of detectable D-Ala-D-Ala-B dipeptide formation suggests an inactive enzyme complex consisting of D-alanine, ADP, Ala-B, and P_i (or Ala-B-PO_3^{2-}).

Thus, there is a variety of ways in which Ala-B can interact with the two D-alanine binding sites of D-Ala-D-Ala ligase, and these are summarized in Scheme II. In case 1, the binding of two D-alanine molecules at the active site occurs in which one D-alanine (N-terminus) is phosphorylated, leading to normal catalytic turnover. In case 2, Ala-B interacts with D-Ala-D-Ala ligase in the presence of ATP to form an initial E·I·ATP (or ADP, P_i) complex or E·I·I·ATP (or ADP, P_i) complex with a binding constant of either 18 or 35 μM, from which enzyme can regain activity by dissociation with a half-life of ca. 2 h. Case 3 occurs in the presence of D-alanine, in which two E·S·I·ATP complexes can form. At low substrate concentrations (case 3a, Scheme II), D-alanine can only compete with Ala-B at the first binding site since the K_m for D-alanine binding is lower at this site than at the second D-alanine binding site [660 μM for the *S. faecalis* enzyme (Neuhaus, 1962)]. Thus, the 2.5-h half-life complex would consist of D-Ala-D-Ala ligase complexed with ADP and perhaps the D-alanyl-PO_3^{2-} acyl phosphate at the first site and Ala-B at the second D-alanine binding pocket. The longer lived complex is only observed at high (20 mM) D-alanine concentrations where D-alanine can now effectively compete with Ala-B at the second binding site [K_m of 10 mM for the *S. faecalis* enzyme (Neuhaus, 1962)], thus forming an enzyme·Ala-B·P_i·ADP·D-alanine complex (case 3b, Scheme II).

Conclusions

Ala-B joins Ala-P as an alanine analogue that induces active site directed, time-dependent inhibition by slow binding equilibria with the alanine racemase from *B. stearothermophilus*. Ala-B is less potent than Ala-P in terms of both K_i and the lifetime of the corresponding E·I′ complex (Table I), and the two analogues probably act by distinct microscopic interactions. Thus, Ala-P induces no change in the PLP chromophore upon racemase inhibition, accumulating as an Ala–P–PLP aldimine in which the dianion of Ala-P appears to be a crucial feature (Copie et al., 1988). By contrast, Ala-B does alter the PLP chromophore, and the structure of the accumulating adduct remains to be determined. Whether a tetrahedral boronate anion [$R-B(OH)_3^-$] adds to the PLP aldimine or some other nuclophile adds to the $-B(OH)_2$ group of Ala-B, a key reactivity is likely to be the electrophilic nature of the boron atom in Ala-B, the feature that is used to advantage in serine protease and β-lactamase inhibition.

Ala-B is also an effective, ATP-dependent, slow binding inhibitor of the next enzyme in the D-alanine pathway, D-Ala-D-Ala ligase, and its behavior is reminiscent of the slow binding features of the (aminoalkyl)phosphinate recently studied with this enzyme (Table I) (Duncan & Walsh, 1988). The D-Ala-D-Ala ligase can be inactivated with a stoichiometric composition of ADP and P_i bound or with D-alanine, ADP, and P_i bound along with Ala-B, with more than one type of complex enabled by the two binding sites on the ligase for D-alanine (Scheme II). It is unclear whether Ala-B, Ala-P, or the (aminoalkyl)phosphinate act as nucleophiles for γ-PO_3^{2-} transfer to yield covalent phosphorylated intermediates, analogous to reaction intermediates, which are stabilized in the ligase active site. Solid-state NMR analysis (^{13}C, ^{11}B, ^{31}P) of specific long-lived complexes may be useful in structural analysis of these unusually long lived species.

Acknowledgments

We are grateful to Dr. Paul Gallop for helpful discussions.

Registry No. **1**, 86595-27-9; **2**, 119414-74-3; **3**, 119414-75-4; **4**, 119414-76-5; **5**, 119414-77-6; EC 5.1.1.1, 9024-06-0; EC 6.3.2.4, 9023-63-6; (dichloromethyl)lithium, 2146-67-0; lithiohexamethyl-

disilazane, 4039-32-1; hexamethyldisilazane, 999-97-3.

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Inhibition of Aminopeptidases by Aminophosphonates†

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ABSTRACT: More than 30 aminophosphonates were synthesized to probe how the structural changes introduced into the phosphonic acid analogue of leucine, a potent inhibitor of cytosolic leucine aminopeptidase (Giannousis & Bartlett, 1987), affect their ability to inhibit cytosolic (EC 3.4.11.1) and microsomal (EC 3.4.11.2) aminopeptidases. Although most of the compounds studied were found to exert only a modest competitive inhibitory effect, nearly every modification of the structure of the phosphonic acid analogue of leucine was reflected in a marked difference in the affinities of these compounds for the two enzymes. [1-Amino-2-(*N*-alkylamino)ethyl]phosphonic acids are effective inhibitors of the microsomal enzyme, acting in a time-dependent manner. Kinetic data obtained for these inhibitors correspond to the mechanism for a biphasic slow-binding inhibition process: $E + I \rightleftharpoons E^* \rightleftharpoons E^*I$, in which the slow initial isomerization of the enzyme is followed by the fast formation of enzyme–inhibitor complex. The most effective inhibitor of this type was [1-amino-2-(*N*-cyclohexylamino)ethyl]phosphonic acid, which has a K_i value of 0.87 μM toward the microsomal aminopeptidase—a value that can be considered as equipotent with bestatin and with leucinal and hydroxamic acids, the strongest known nonpeptide inhibitors of this enzyme.

Aminopeptidases are a group of zinc-containing exopeptidases that catalyze the hydrolysis of N-terminal peptide bonds in polypeptide chains. Among these enzymes, leucine aminopeptidase has been the most extensively studied. However, by comparison with carboxypeptidases (also zinc-containing enzymes), structural and mechanistic information about this class of proteases is less extensive.

The biological importance of aminopeptidases is indicated by the fact that aminopeptidase activity has been detected in many mammalian tissues, organs, and bodily fluids and in cell membranes and plants (DeLange & Smith, 1971; Aoyagi et al., 1976).

The development of synthetic inhibitors of proteases is an active field of research that has provided insight into the nature of enzyme–substrate interactions during catalysis. Effective inhibitors reported for aminopeptidases include aminoketones

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