ATP-Dependent Inactivation and Slow Binding Inhibition of *Salmonella typhimurium* D-Alanine:D-Alanine Ligase (ADP) by (Aminoalkyl)phosphinate and Aminophosphonate Analogues of D-Alanine†

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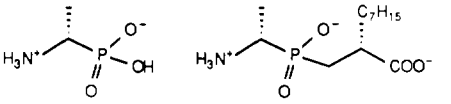
ABSTRACT: In *Salmonella typhimurium*, D-alanine:D-alanine ligase (ADP) (EC 6.3.2.4) is the second enzyme in the three enzyme D-alanine branch pathway of peptidoglycan biosynthesis. The interaction of this enzyme with a possible transition-state analogue, the (aminoalkyl)phosphinate D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid [Parsons et al. (1987) *Abstracts of Papers*, 193rd National Meeting of the American Chemical Society, Denver, CO, MEDI 63, American Chemical Society, Washington, DC], has been studied. This compound is a potent active site directed inhibitor and is competitive with D-alanine (K_i = 1.2 μM); it exhibits time-dependent inhibition in the presence of ATP. Kinetic analysis revealed a rapid onset of steady-state inhibition (k_{on} = 1.35 × 10^4 M^{-1} s^{-1}) followed by slow dissociation of inhibitory complex(es) with a half-life of 8.2 h. The inhibitory complex was shown to consist of E···I···ATP in equilibrium with E···I, P_i, and ADP. Similar time-dependent inhibition was also observed with D-(1-aminoethyl)phosphonic acid (D-Ala-P) (K_i = 0.5 mM; k_{on} = 27 M^{-1} s^{-1}; $t_{1/2}$ for regain = 1.73 min) but not with D-(1-aminoethyl)phosphinic acid, which behaved as a simple competitive inhibitor (K_i = 0.4 mM). The mechanism of inhibition is discussed in the light of the precedents of glutamine synthase inhibition by methionine sulfoximine and phosphinothricin.

In the biosynthesis of the peptidoglycan layer of the bacterial cell wall, three enzymes comprise the D-alanine branch, namely, alanine racemase (EC 5.1.1.1), D-alanine:D-alanine ligase (ADP) (EC 6.3.2.4), and the D-Ala-D-Ala adding enzyme (EC 6.3.2.15) [for a review, see Neuhaus and Hammes (1981)]. The racemase and ligase are targets for naturally elaborated antibacterial agents such as *O*-carbamyl-D-serine (Lynch & Neuhaus, 1966) and D-cycloserine (Neuhaus & Lynch, 1964), respectively. Although of negligible to small current value clinically, their isolation and characterization focused attention on racemase and ligase as killing sites (Park, 1958). We have recently described studies in the molecular biology and enzymology of alanine racemase and the mode of action of synthetic antibacterials such as β-fluoro-D-alanine (Badet et al., 1984; Roise et al., 1984) and (1-aminoethyl)-phosphonate (Ala-P) (Badet et al., 1986) on alanine racemases from Gram-negative and Gram-positive bacteria.

By contrast, much less is known about the second target enzyme, the D-Ala-D-Ala ligase. Partial purification and extensive kinetic studies on the ligase from the Gram-positive *Streptococcus faecalis* have been detailed some time ago (Neuhaus, 1962a,b; Neuhaus & Lynch, 1964). The cloning and DNA sequence analysis of the *Escherichia coli ddl* gene, which encodes D-Ala-D-Ala ligase, have been described (Robinson et al., 1986). Similarly, the *Salmonella typhimurium ddlA* gene was recently isolated in these laboratories, and we have purified the cloned *Salmonella* ligase to homogeneity (Daub et al., 1988).

In this paper we report time-dependent inactivation of this ligase with D-Ala-P (**1**) and with the (aminoalkyl)phosphinate **2**, recently described by Parsons et al. (1987). We demonstrate the need for ATP in the progressive loss of activity and interpret these results in the light of slow binding inhibition precedents (Morrison & Walsh, 1987).



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Materials and Methods

Materials. ATP, D-Ala, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and Sephadex G-50 were purchased from Sigma. [^{14}C]ATP and [γ-^{32}P]ATP were purchased from Amersham. D- and L-Ala-P were from Fluka. The pyruvate kinase–lactate dehydrogenase mixture (PK–LDH) (1:1 ratio of activities), phosphoenolpyruvate, and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Boehringer Mannheim Biochemicals. D- and L-(1-aminoethyl)phosphinic acids were obtained as gifts from Dr. J. Dingwall, Ciba-Geigy, Basel. The D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid was a gift from Dr. A. A. Patchett, Merck Research, Rahway, NJ.

Preparation of D-*Ala*-D-*Ala Ligase.* Plasmid-encoded D-Ala-D-Ala ligase was purified from *S. typhimurium* DB7000/pDS4 as described in Daub et al. (1988). The final pool was judged homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had a final specific activity of 16 μmol min^{-1} mg^{-1} by the PK–LDH coupled assay.

Protein concentrations were estimated by the method of Bradford (1976).

Assays. D-Ala-D-Ala ligase was assayed spectrophotometrically by coupling the hydrolysis of ATP to the PK–LDH reactions as described previously (Daub et al., 1988). The

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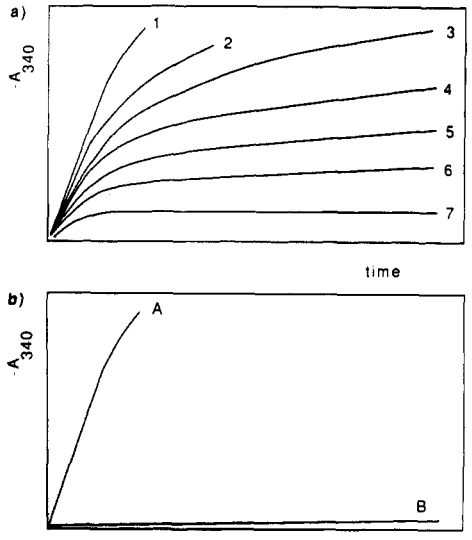


FIGURE 1: (a) Progress curves of D-Ala-D-Ala ligase assays in the presence of (aminoalkyl)phosphinate **2**. The ordinate and abscissa indicate the time course and the decrease in A_{340} during the reaction, respectively. PK–LDH coupled assay mixtures contained 4.5 μg of enzyme and inhibitor at the following final concentrations: (1) 0, (2) 0.5, (3) 0.8, (4) 1.0, (5) 1.4, (6) 2.5, and (7) 3.0 μM. (b) Progress curves of assays after preincubation of ligase with inhibitor, as described in text: (A) no ATP; (B) with ATP.

Table I: Kinetic Parameters for Inhibition by Merck (Aminoalkyl)phosphinate

[I] (μM)	k_{obsd} (min^{-1})	k_{on} (μM^{-1} min^{-1})
1.0	0.812	0.812
1.4	1.273	0.909
2.0	1.760	0.880
2.5	1.989	0.994
3.0	2.145	0.715
3.3	2.494	0.756
5.0	3.098	0.620

decrease in absorbance at 340 nm as NADH is consumed was measured at 37 °C on a Perkin-Elmer λ5 instrument.

RESULTS

Inhibition of D-*Ala*-D-*Ala Ligase by (Aminoalkyl)phosphinate.* The inhibitory effect of the (aminoalkyl)phosphinate **2** on D-Ala-D-Ala ligase was observed when the enzyme (115 pmol) was added to PK–LDH coupled assay mixtures containing D-Ala, ATP, and **2**. The resulting progress curves are shown in Figure 1a, where the clear nonlinearity observed suggests a time-dependent process in which the initial rate of D-Ala processing is reduced to a lower steady-state rate. Progress curves were obtained over a range of D-Ala and inhibitor concentrations, and analysis of the initial rates of reaction by the method of Dixon (1953) (data not shown) showed that the (aminoalkyl)phosphinate was an inhibitor of the ligase, competitive with D-Ala, with a K_i = 1.2 μM.

The slow onset of inhibition was observed for 3–4 min during an assay. The rate of approach to steady state, k_{obsd}, was determined by measuring the reaction velocity at various times (Table I). From eq 1 (Morrison & Walsh, 1987)

$$v = v_s + (v_0 - v_s) \exp(-k_{obsd}t) \quad (1)$$

where v_s, v_0, and v are the steady-state, initial, and time t velocities; a plot of ln $[(v - v_s)/(v_0 - v_s)]$ vs t will be linear and have a slope equal to $-k_{obsd}$. The resulting plot is shown in Figure 2a. From the values of k_{obsd} obtained at various inhibitor concentrations, the rate of formation of the initial EI complex, k_{on} ($=k_{obsd}/[I]$), was calculated to be 1.35×10^4 M^{-1} s^{-1}. Figure 2b is a plot of $1/k_{obsd}$ vs $1/[I]$, which is linear

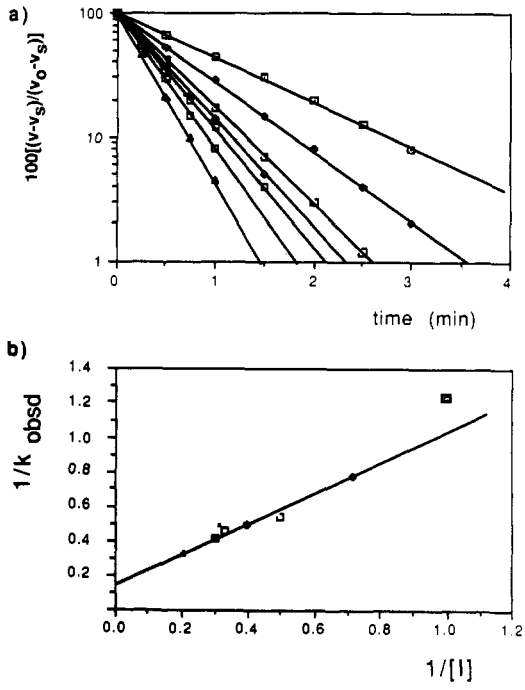


FIGURE 2: Kinetics of onset of slow-binding inhibition by Merck (aminoalkyl)phosphinate. (a) Plot of ln $100[(v - v_s)/(v_0 - v_s)]$ vs t at several inhibitor concentrations: (▣) 1.0, (◆) 1.4, (❑) 2.0, (◇) 2.5, (*□) 3.0, (□) 3.3, and (▲) 5.0 μM. k_{obsd} was calculated from the slopes of the lines. (b) From this plot of $1/k_{obsd}$ vs $[I]$, k_{inact} was calculated from the intersect with the abscissa.

over the range used in this experiment. From the intersect with the y axis, a k_{inact} (the rate constant for the onset of slow inhibition) of 6.9 min^{-1} was observed, yielding $t_{1/2}$ for inactivation of 0.1 min.

ATP Requirement for Time-Dependent Inhibition of D-*Ala*-D-*Ala Ligase by (Aminoalkyl)phosphinate.* In order to assess the rate at which fully inhibited enzyme regains activity upon removal of the inhibitor, D-Ala-D-Ala ligase (25 pmol) was incubated with **2** (20 μM) for 1 h at room temperature before assaying residual activity. The inhibitor was effectively removed from the enzyme by 50-fold dilution in the 1-mL assay, but the resulting progress curve (Figure 1b, A) showed no effect due to the preincubation; i.e., there was no detectable loss of catalytic activity. When a similar preincubation was carried out in the presence of **2** and ATP (5 mM), the progress curve of this assay (Figure 1b, B) showed almost full loss of activity; only 2.5% of the original activity remained. Regain of activity was not detectable on a steady-state time scale. Preincubation of ligase, (aminoalkyl)phosphinate, and ADP (5 mM) did not result in inhibition.

Regain of Catalytic Activity after Incubation of Ligase with (Aminoalkyl)phosphinate and ATP. The regain of catalytic activity of fully inhibited enzyme was monitored over several hours in the following way: D-Ala-D-Ala ligase (5 nmol) was inactivated by incubation overnight at 4 °C in 100 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 10 mM KCl, 250 μM (aminoalkyl)phosphinate, and 1 mM ATP in a final volume of 0.3 mL. In separate experiments, the ATP was uniformly labeled with ^{14}C (15 nCi/nmol) or γ-labeled with ^{32}P (20 nCi/nmol). At this stage, the residual enzyme activity was estimated to be <3% of starting activity, although this was difficult to measure accurately as the concentration of inhibitor in the assay was now significant. The inhibited enzyme was subjected to gel filtration chromatography on Sephadex G-50 to remove unbound (aminoalkyl)phosphinate and ATP. Protein-containing fractions were pooled and assayed over a

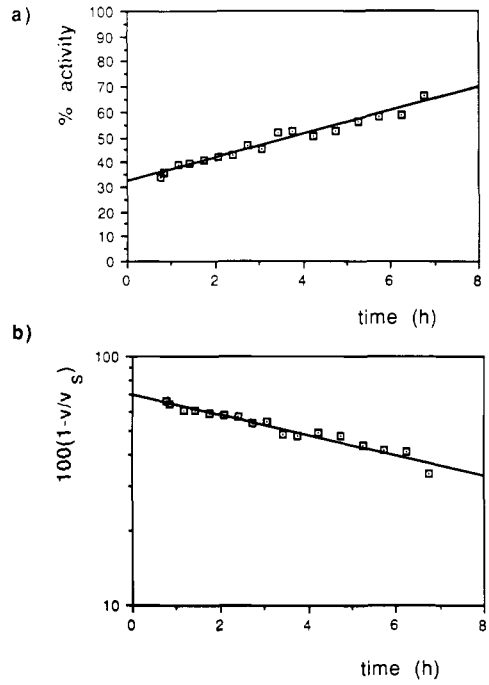


FIGURE 3: Regain of catalytic activity of D-Ala-D-Ala ligase after gel filtration of an incubation mix of ligase with Merck (aminoalkyl)phosphinate and ATP (as described in the text). (a) Plot showing the recovery of catalytic activity over an 8-h time period. (b) Plot of ln $[100(1 - v/v_s)]$ vs t, where v = velocity at time t and v_s = final velocity reached; the slope is equal to $-k_{rgn}$. In each case, $t = 0$ is defined as the time at which the sample was loaded onto the gel filtration column; enzyme-containing fractions eluted at $t = 40$; the first measurement of enzyme activity was at $t = 45$.

number of hours (Figure 3a) and until the activity had returned to 100% of the activity of a control sample, which had been incubated in the absence of inhibitor but had been through the gel filtration procedure. It is immediately obvious by extrapolation from Figure 3a that prior to gel filtration the enzyme had in fact retained approximately 33% of the starting activity during the preincubation. It remains unclear to us why this should be so, since 250 μM inhibitor is over 200 times the K_i. In the smaller scale experiments described above aimed at determining regain by dilution and assay, the enzyme was clearly fully inhibited; the only real difference between these two sets of experiments is that the protein concentration is much lower in the smaller scale experiments. In a number of experiments, the preincubation was carried out for 1, 6, 16, and 72 h; each time the first rate determined upon gel filtration was found to be in the 25–35% range (compared with similarly treated control enzyme).

The rate of regain of catalytic activity, k_{rgn}, was determined from a plot of ln $[100(1 - v/v_s)]$ vs t, where v = velocity at time t and v_s = final velocity reached (Figure 3b). The plot was linear over the first 8 h, and from the slope (equal to $-k_{rgn}$), k_{rgn} was determined to be 1.4×10^{-3} min^{-1}, yielding a $t_{1/2}$ of 8.2 h.

Stoichiometry of Label Bound to Enzyme. Following gel filtration, the stoichiometry of binding of the label to D-Ala-D-Ala ligase was determined by correlating the counts bound to the enzyme with the amount of enzyme present, determined by the micro-Bradford assay. In an experiment with [^{14}C]-ATP, 0.56 mol of label was bound to 1 mol of enzyme. A similar stoichiometry was found with the ^{32}P-labeled ATP. However, if one assumes from the data on reactivation above that prior to gel filtration the enzyme retained 33% activity and that the label is bound only to inactive enzyme complexes, then it follows that the stoichiometry is actually 0.83 mol of label bound per mol of inactive ligase.

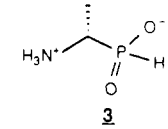
At this time, we are unable to determine the stoichiometry for the inhibitor in the inhibited enzyme complex due to the unavailability of labeled (aminoalkyl)phosphinate.

Nature of the Species Bound to Inactive Enzyme. A sample from the gel filtration pool was denatured by heating (100 °C, 2 min), chilled on ice (30 min), and then centrifuged for 10 min (microfuge). Under these conditions, ATP was found to be stable to hydrolysis. An aliquot of the supernatant was spotted onto a PEI–cellulose thin-layer chromatography (TLC) plate and developed in 1 M potassium phosphate (pH 3.4). After being dried, the plate was autoradiographed and regions corresponding to the location of any spots were cut out and counted after being placed in 10 mL of Liquiscint. Samples prepared in this way had to be counted many times in order to produce a statistically significant value for the radioactivity present in a spot.

^{14}C-Labeled enzyme produced two spots on autoradiography, which corresponded to ATP and ADP. Approximately 68% of the counts were in ATP and 32% in ADP. With ^{32}P-labeled enzyme, two spots were also identified, this time corresponding to ATP and P_i. The counts were 59% in ATP and 41% in P_i. The P_i may have been derived from a phospho(aminoalkyl)phosphinate adduct too labile to survive in solution or to survive the conditions used to prepare the samples for TLC. These observations imply that a proportion of the inactive enzyme has ATP and presumably inhibitor bound (since in a control experiment where only [γ-^{32}P]ATP and ligase were incubated, no enzyme-bound label was detected after gel filtration) and the remainder has ADP and the presumed phospho(aminoalkyl)phosphinate bound.

Is Activity Regain Due to Loss of ATP, ADP, or Both from the Inactive Enzyme? In order to address this question, half of the ^{14}C-labeled gel filtration pool was subjected to a second gel filtration, 7 h after the first. The peak protein fraction was retained and analyzed in a way similar to that of the first pool. The stoichiometry of labeling was now 0.28 mol of label bound per mole of ligase (since the enzyme now exibited 64% of full activity, this corresponds to 0.77 mol of label per mole of inactive enzyme). TLC revealed that the counts remained in both ATP and ADP and were apportioned 62% ATP and 38% ADP. This result suggests that ATP and ADP are being lost from the enzyme at the same, or approximately the same, rate; recovery is clearly not due to the rapid loss of one or the other. This result was confirmed in a further experiment in which ^{32}P-labeled enzyme was subjected to a second gel filtration step.

Kinetic Analysis of Inhibition by (1-Aminoethyl)phosphinate. The simple phosphinic acid **3** was tested as an inhibitor of ligase. Only D-(1-aminoethyl)phosphinate was active; the



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L isomer had no effect on rates of D-Ala processing. **3** was competitive with D-Ala, and a Dixon plot (data not shown) yielded a value for the K_i of 0.4 mM. No time-dependent reduction in the rate was seen, with or without ATP. Although this compound is structurally similar to the Merck inhibitor described above (it lacks the heptylpropionic acid moiety), it must be incapable of bringing about the slow transition to an E*I complex.

Kinetic Analysis of Inhibition by D-Ala-P. In the presence of the phosphonate D-Ala-P, progress curves monitoring the

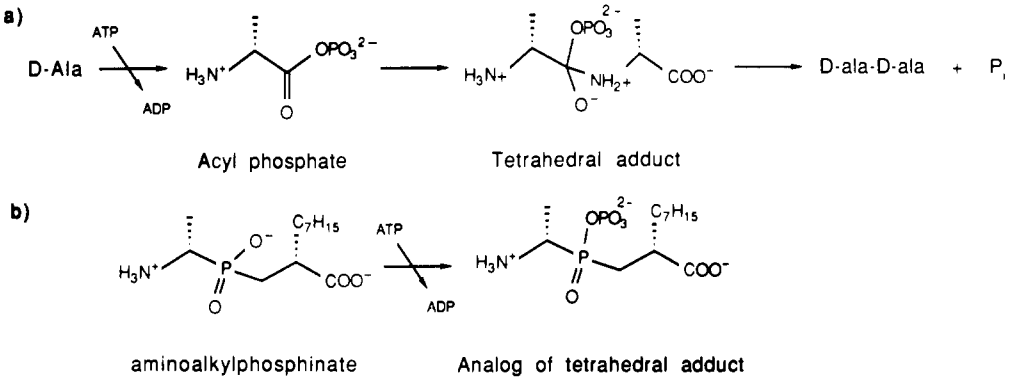


FIGURE 4: (a) Proposed reaction mechanism of D-Ala-D-Ala ligase, based on an acyl phosphate intermediate, by analogy with the observed acyl phosphate intermediate γ-glutamyl-PO_3^{2-} of glutamine synthase. (b) Proposed mechanism of inhibition of D-Ala-D-Ala ligase by the Merck (aminoalkyl)phosphinate.

Table II: Kinetic Parameters for Inhibition by D-Ala-P

[I] (mM)	k_{obsd} (min^{-1})	k_{on} (mM^{-1} min^{-1})
0.5	0.992	1.984
1.0	1.458	1.458
2.0	3.702	1.851
4.0	4.766	1.191

PK–LDH coupled reaction of the ligase with D-Ala show a pattern similar to that in Figure 1a, suggesting that in the presence of ATP this compound inhibits the enzyme with slow binding kinetics, analogous to the Merck (aminoalkyl)phosphinate. L-Ala-P has no effect on ligase. From initial rates of D-Ala processing (data not shown), a Dixon plot yields a value of 0.5 mM for the D-Ala-P K_i and also shows that D-Ala-P is a competitive inhibitor of ligase. This value is 400-fold higher than the K_i obtained with the (aminoalkyl)phosphinate.

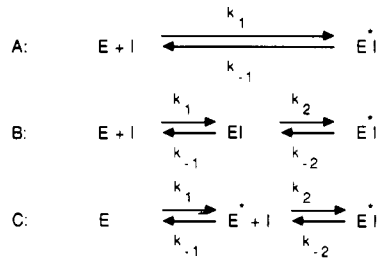
From reaction velocities at various times during the onset of inhibition, k_{obsd} was calculated for a range of inhibitor concentrations (Table II). The average k_{on} was then calculated to be 27 M^{-1} s^{-1}. The limiting k_{inact} was 18.2 min^{-1}, and the $t_{1/2}$ for activity loss was 0.04 min.

An assay mixture was monitored at 340 nm in the absence of D-Ala and in the presence of D-Ala-P (5 mM). Under these conditions, no turnover of ATP was seen.

Regain of Activity Following Inhibition by D-Ala-P. Incubation of D-Ala-D-Ala ligase with D-Ala-P in the absence and presence of ATP showed that this compound also requires ATP to stimulate inhibition. A 25-pmol aliquot of D-Ala-D-Ala ligase was incubated in the presence of D-Ala-P (10 mM) and ATP (1 mM) for 1 h at room temperature, by which time the enzyme was fully inactive. Inhibited enzyme was then assayed (100-fold dilution of inhibitor). In the case of this inhibitor, the rate of regain of catalytic activity was rapid enough to be measured over a number of minutes. A plot of ln $[100(1 - v/v_s)]$ vs time yielded a straight line with slope ($=-k_{rgn}$) equal to 0.4 min^{-1}, from which a $t_{1/2}$ of 1.73 min was calculated.

Gel Filtration of D-Ala-P-Inhibited Ligase. Five nanomoles of D-Ala-D-Ala ligase was incubated with D-Ala-P (20 mM) and [γ-^{32}P]ATP (1 mM, 20 nCi/nmol) for 1 h at room temperature. This mixture was then subjected to gel filtration chromatography as described above for the (aminoalkyl)-phosphinate-treated enzyme. Due to the very fast rate of activity regain from this inhibitor, the enzyme was >90% active by the time it was recovered from the column. Therefore, no information was gained about the stoichiometry of binding of the label to the inhibited enzyme, and only a low number of counts were recovered. An aliquot of this enzyme was denatured and subjected to TLC. Only a single faint spot corresponding to P_i was observed; no ATP was seen. These results again are consistent with the hydrolysis of ATP to form a transient phospho-D-Ala-P adduct.

Scheme I



DISCUSSION

D-Ala-D-Ala ligase catalyzed formation of D-Ala-D-Ala most likely proceeds by a mechanism that involves carboxyl activation of the first D-Ala, generating the acyl phosphate intermediate D-alanyl-PO_3^{2-}, which suffers nucleophilic attack by the amide nitrogen of the second D-Ala (Figure 4a). Patchett and his colleagues recently reported the synthesis of a number of phosphinic acid analogues of D-alanyl-D-alanine as potential analogues of transition states or intermediates along this catalytic pathway (Parsons et al., 1987). Among the more potent inhibitors synthesized was compound **2**, D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid, where the methyl group corresponding to the second D-alanyl residue is replaced by a heptyl side chain. This analogue showed an IC_{50} of 4 μM in studies with crude extracts of the *S. faecalis* D-Ala-D-Ala ligase. Through the kind gift of compound **2** from the Merck group, we began to assess its potency with the *Salmonella* ligase. When inhibitor **2** was added to continuous spectrophotometric assays, a dramatic effect on the rate of D-Ala processing was observed; the pattern of the progress curves was prototypic for the class of slow binding inhibitors (Morrison & Walsh, 1987), in which progressive time-dependent inhibition arises from the slow establishment of binding equilibria between enzyme and inhibitor and where slow off-rates dominate the kinetics and produce long-lived noncovalent complexes. Several mechanisms have been proposed to account for time-dependent inhibition (Scheme I). In general, mechanism B applies; we propose below that from the evidence described at this time a more complicated variant of this basic equation is responsible for the kinetics observed with ligase and the (aminoalkyl)phosphinate.

Kinetic analysis revealed a K_i of 1.2 μM and k_{inact} of 6.9 min^{-1} for compound **2**. As the apparent K_m for D-Ala in turnover is 1.4 mM, the (aminoalkyl)phosphinate has a K_i/K_m ratio of 1/1100, reflecting a substantial increase in affinity

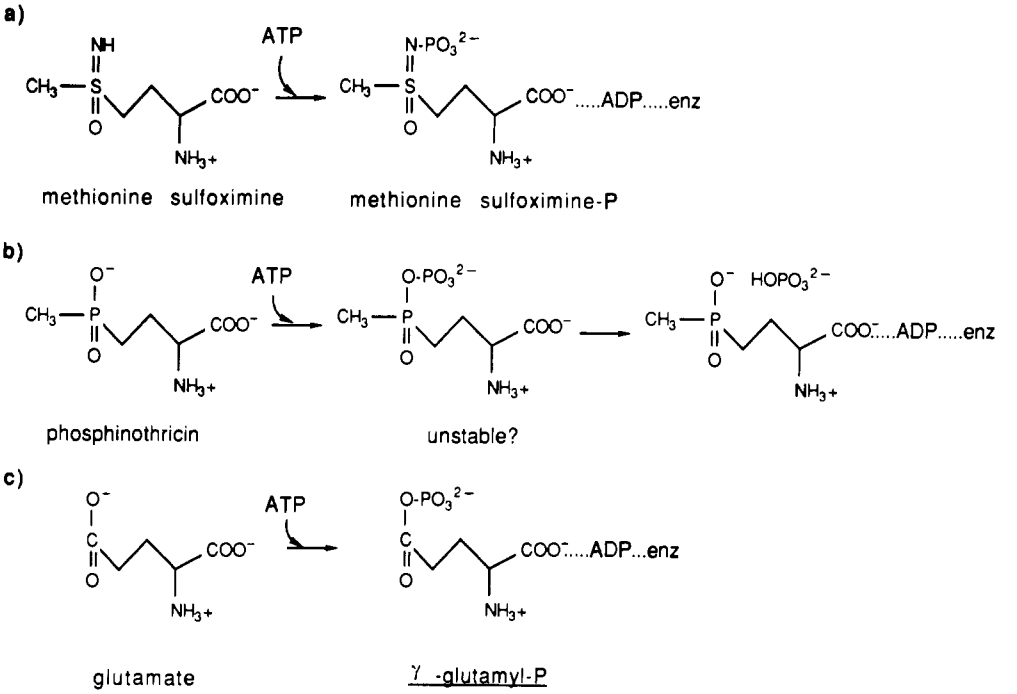


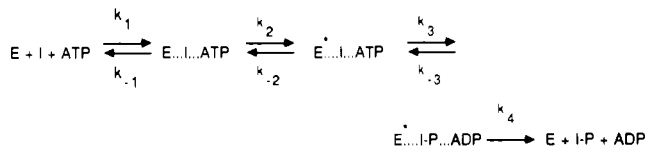
FIGURE 5: (a) Mechanism of inhibition of *E. coli* glutamine synthase by methionine sulfoximine. (b) Mechanism of inhibition of the same enzyme by phosphinothricin. (c) Normal reaction catalyzed by glutamine synthase.

as compared with substrate. The bimolecular rate constant for the rapid formation of the initial EI complex (k_{on}) was found to be 1.35×10^4 M^{-1} s^{-1}. Initial EI complex formation usually occurs with a rate constant similar to that for ES formation and in the range of 10^5–10^7 M^{-1} s^{-1} (Fersht, 1984). The slightly lower value reported may be an indication that more than one step is involved in the process of formation of the initial EI complex, but it is not definitive evidence.

The participation of ATP in the formation of a long-lived EI complex was clearly demonstrated in experiments where enzyme was preincubated with inhibitor. Only in the presence of ATP was any effect seen. Dissociation of EI complex could only be detected over a number of hours after gel filtration. It was found that the complex dissociates slowly with a half-life of over 8 h. Most slow binding inhibitors that have been studied regain activity with $t_{1/2}$ in the minutes range, but several have been described with half-lives of many hours (Morrison & Walsh, 1987). A notable few have exceptionally long half-lives; it was recently shown in this laboratory, for example, that L-Ala-P dissociates from *Bacillus stearothermophilus* alanine racemase with a remarkably long 25-day half-life (Badet et al., 1986). Analysis of the species bound to inactive ligase revealed that the enzyme turns over ATP to ADP and P_i; the latter may be derived from a labile form of phosphorylated inhibitor. There would therefore be an E···I···ATP complex in equilibrium with an E···I–P···ADP complex, with an equilibrium constant close to 1. The k_{rgn} calculated for the dissociation of enzyme from inhibitor must therefore represent the sum of two processes, namely, release of ATP and I from the E···I···ATP complex and release of ADP, P_i, and I from the E···I–P···ADP complex. Thus, the process of inhibition by (aminoalkyl)phosphinate involves multiple steps, and several equilibria contribute to the overall efficacy of this compound. In Scheme II we describe a possible series of equilibrium steps. Under this scheme, $K_i = k_{-1}/k_1$, $k_{on} = k_2/(K_i + [I]) + k_{-2}/[I]$, and $k_{rgn} = k_4 + k_{-1}$.

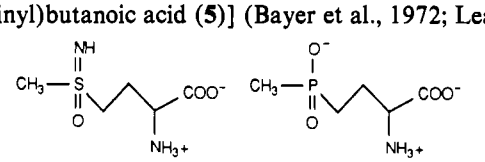
Time-dependent inactivation of D-Ala-D-Ala ligase was also observed with D-Ala-P. D-Ala-P is not as potent a ligase inhibitor; the K_i observed (0.5 mM) is approximately one-third

Scheme II



the apparent K_m for D-Ala, although D-Ala-P does bring about steady-state inhibition more rapidly than (aminoalkyl)phosphinate (k_{inact} = 18.2 min^{-1}). The very low bimolecular rate constant of 27 M^{-1} s^{-1} (10^3–10^5-fold lower than typical k_{on} rates) is very convincing evidence that more than one step precedes formation of the initial EI complex (Morrison & Walsh, 1987). Activity was regained from fully D-Ala-P-inhibited enzyme with a half-life of less than 2 min. Evidence was obtained that ATP fragmentation and possible phosphorylation of D-Ala-P is involved in the mechanism of inhibition, but no enzyme-bound ATP was detected. This probably suggests that only an E···phospho-D-Ala-P···ADP complex is responsible for inhibition in this case. It is likely that inhibition by D-Ala-P follows the same overall outline as shown in Scheme II, except that the equilibria are shifted in favor of the E···phospho-I···ADP complex.

At this juncture, the precedents of methionine sulfoximine (**4**) (Rowe et al., 1969; Manning et al., 1969) and, especially, the alkylphosphinate phosphinothricin [2-amino-4-(methylphosphinyl)butanoic acid (**5**)] (Bayer et al., 1972; Leason et

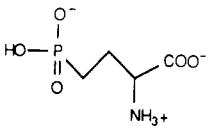


4 **5**

al., 1982) as time-dependent inactivators of glutamine synthase seem relevant (Colanduoni & Villafranca, 1986). Both glutamine synthase and D-Ala-D-Ala ligase catalyze amide formation from a substrate carboxylate, ATP, and an amine. Methionine sulfoximine and phosphinothricin inactivate glutamine synthase in an ATP-dependent process leading to

phosphorylation of the sulfoximine nitrogen or (presumably) the phosphinate oxygen to yield a methionine sulfoximine-PO_3^{2-} or possibly a phosphinothricin-PO_3^{2-}···ADP product complex that is noncovalently attached to glutamine synthase but so tightly bound that they do not dissociate over a period of hours (Rowe et al., 1969) or minutes (Colanduoni & Villafranca, 1986), respectively (Figure 5). The methionine sulfoximine-PO_3^{2-} is thought to be a structural analogue of the normal intermediate γ-glutamyl-PO_3^{2-}. A similar acyl phosphate, D-alanyl-PO_3^{2-}, seems a likely intermediate in D-Ala-D-Ala ligase catalysis (Figure 4a).

While the alkylphosphinate phosphinothricin is an ATP-dependent inactivator of glutamine synthase, the corresponding phosphonate **6** (2-amino-4-phosphonobutyric acid) also binds



6

to this enzyme in the presence of ATP, but it does not bring about inactivation; instead, it is a substrate, presumably undergoing phosphorylation and release (Colanduoni & Villafranca, 1986). In contrast, the phosphonate D-Ala-P does inactivate D-Ala-D-Ala ligase, albeit with a much lower potency than the (aminoalkyl)phosphinate **2** and a 300-fold faster release rate of products. On the basis of these precedents, and from the evidence presented above, we propose in Figure 4b a mechanism for inhibition of D-Ala-D-Ala ligase by the (aminoalkyl)phosphinate in which the phosphinate oxygen acts as nucleophile toward the γ-P of ATP and the resultant phosphorylated (aminoalkyl)phosphinate mimics the tetrahedral adduct that would form when the amine nitrogen of the second condensing D-Ala attacks the D-alanyl-PO_3^{2-} acyl phosphate reaction intermediate. Unlike the normal acyl phosphate species, which would rapidly react with the amino group of the second D-ala molecule, the phosphinophosphate ester would remain tightly bound and only slowly dissociating. We note that, in the glutamine synthase and the D-Ala-D-Ala ligase cases, the putative phosphinophosphate esters have not been detected directly but only P_i and phosphinate on derivatization or slow release. The hydrolytic stability of such species off these enzymes has yet to be determined. In contrast, the presumably cognate slow binding inhibitor methionine sulfoximine-PO_3^{2-} is stable and isolable from stoichiometrically inhibited glutamine synthase.

In sum, we describe for the first time slow-binding inhibition of D-Ala-D-Ala ligase, a known target for the antibacterial agent D-cycloserine. The onset of inhibition is ATP dependent and reflects buildup of an equilibrium between an inhibitor···ATP···enzyme complex and a phosphoinhibitor··· ADP···enzyme complex, both of which dissociate exceedingly slowly, in analogy to phosphinothricin behavior with glutamine synthase. Further exploration of the mode of action of **1** and **2** should also shed light on the catalytic mechanism of ligase. Finally, we now have evidence that Ala-P (**1**) can be a slow binding, nondissociating inhibitor of the first enzyme, alanine racemase, and a slow binding, rapidly dissociating inhibitor of the second enzyme, D-Ala-D-Ala ligase, of the three enzyme D-alanine branch of peptidoglycan biosynthesis in bacteria.

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