Synthesis and evaluation of inhibitors of bacterial D-alanine:D-alanine ligases

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Background: D-Alanine:D-alanine ligase is essential for bacterial cell wall synthesis, assembling one of the subunits used for peptidoglycan crosslinking. The resulting aminoacyl-D-Ala-D-Ala strand is the Achilles' heel of vancomycin-susceptible bacteria; binding of vancomycin to this sequence interferes with crosslinking and blocks cell-wall synthesis. A mutant enzyme (VanA) from vancomycin-resistant *Enterococcus faecium* has been found to incorporate α-hydroxy acids at the terminal site instead of D-Ala; the resulting depsipeptides do not bind vancomycin, yet function in the crosslinking reaction. To investigate the binding specificity of these ligases, we examined their inhibition by a series of substrate analogs.

Results: Phosphinate and phosphonate dipeptide analogs (which, after phosphorylation by the enzyme, mimic intermediates in the ligation reaction) were prepared and evaluated as reversible inhibitors of the wild-type ligases DdlA and DdlB from *Escherichia coli* and of the mutant enzyme VanA. K_i values were calculated for the first stage of inhibitor binding according to a mechanism in which inhibitor competes with D-Ala for both substrate binding sites. DdlA is potently inhibited by phosphinates but not by phosphonates, while DdlB and VanA show little discrimination; both series of compounds inhibit DdlB strongly and VanA weakly.

Conclusions: VanA has greatly reduced affinity for all the ligands studied. The relative affinities of the inhibitors in the reversible binding step are not, however, consistent with the substrate specificities of the enzymes. We propose a mechanism in which proton transfer from the attacking nucleophile to the departing phosphate occurs directly, without intervention of the enzyme.

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Introduction

The enzyme D-alanine:D-alanine ligase (E.C. 6.3.2.4) carries out a key step in bacterial cell wall biosynthesis by establishing the peptide linkage that ultimately provides the site of transacylation when the peptidoglycan framework is crosslinked. These enzymes are potently inhibited by phosphinate dipeptides (such as compound **1**, Fig. 1), which are phosphorylated in the active site by ATP to produce mimics (compound **P-1**; Fig. 1) of the tetrahedral intermediate in the acylation mechanism (Fig. 1) [1, 2]. The proposal that phosphinate dipeptides inhibit the ligase via this mechanism was confirmed by the recent structural determination by Knox and coworkers [3] of the complex formed on combination of the *Escherichia coli* enzyme with the D-Ala-D-Ala-phosphinate **1a** and ATP, which produces the mixed anhydride **P-1a** and ADP in the active site. The universality of this inhibition mechanism has, however, been questioned by Al-Bar *et al.* [4], who found no kinetic role for ATP in inhibition of the *E. coli* Ddl enzyme by a phosphonate analog related to **1a** (with R = heptyl).

The D-stereochemistry of the alanine residues of the peptidoglycan allows them to escape the action of most proteases and provides a unique recognition element for the bacterial enzymes that modulate the crosslinking process. These residues also form the recognition site of the vancomycin class of glycopeptide antibiotics, which interfere with bacterial cell wall biosynthesis by binding to the Lys-D-Ala-D-Ala moiety and interfering with the assembly and crosslinking processes. Emergence of resistance to these antibiotics is the result of a dramatic alteration in this pathway, as Walsh and coworkers [5] have discovered. Not only is a ligase of altered specificity required for production of a dipeptidyl unit that is not recognized by the antibiotic, (a D-Ala-D-α-hydroxybutyrate depsipeptide, containing an ester linkage instead of an amide linkage), but changes in upstream enzymes are necessary to provide the altered substrates.

The mutant ligase from a resistant strain of *Enterococcus faecium*, VanA, has been studied by Walsh and coworkers [6,7] and found to have significantly reduced affinity for D-Ala compared to wild-type enzymes (Table 1). It also shows reduced selectivity for D-Ala compared to amino acids with larger hydrophobic sidechains, and, most significantly, it accepts α-hydroxy acids at the second (nucleophilic) site; wild-type ligases are not known to accept hydroxy acids at the second site. To match the altered specificity of the mutant ligase, we synthesized some new dipeptidyl phosphorus analogs (see Fig. 1) and evaluated them as inhibitors of both the wild-type and the mutant ligases. In addition to the known D-Ala-D-Ala phosphinate **1a**, we synthesized the phosphinates corresponding to D-Ala-D-Leu (compound **1b**) and D-Ala-D-Phe (compound **1c**) and the phosphonates corresponding to D-Ala-D-lactate (compound **2a**) and D-Ala-D-α-hydroxybutyrate

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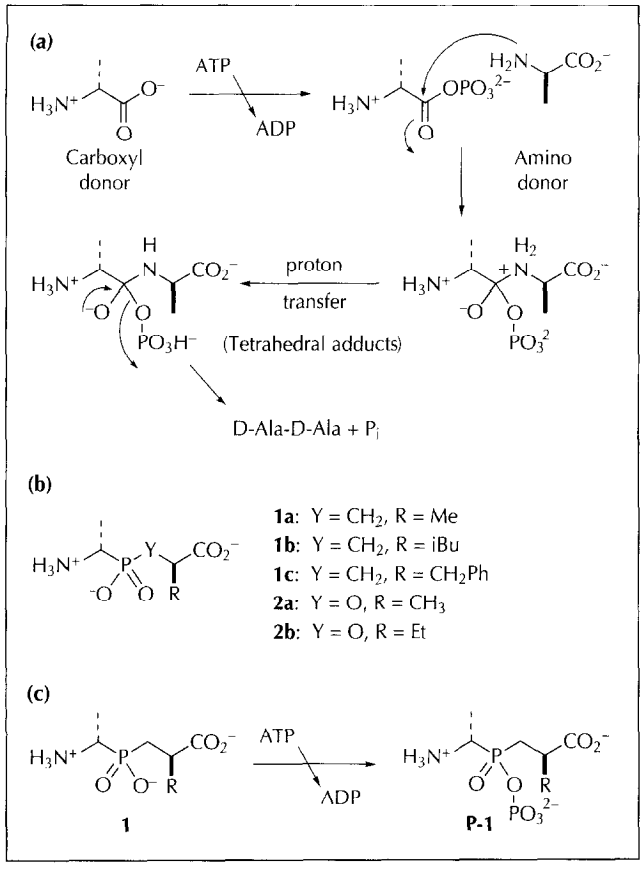

Fig. 1. Mechanism and inhibition of D-Ala:D-Ala ligase. **(a)** Mechanism of the ligase reaction. Phosphorylation of the carboxyl donor is followed by nucleophilic attack by the amino donor to give a tetrahedral adduct; tautomerization by proton transfer leads to an intermediate that loses inorganic phosphate (P_i) and forms the D-Ala-D-Ala dipeptide. **(b)** Structures of phosphinate (**1**) and phosphonate (**2**) inhibitors. **(c)** Mechanism of phosphinate inhibition. Phosphorylation of the phosphinate **1** leads to the mixed anhydride **P-1**, a mimic of the tetrahedral intermediate for dipeptide formation (see (a), above); the phosphonates **2a** and **2b** are expected to react the same way.

(compound **2b**). We studied the phosphonate compounds to examine the specificity of the VanA ligase for ester bond formation.

Kinetic analysis of D-Ala-D-Ala ligase is not straightforward. The two amino acids to be joined occupy distinct binding sites on the enzyme that have different affinities for D-Ala. The Michaelis constants observed for the first

Table 1. Kinetic constants for D-Ala-D-Ala ligases.[a]

Bacterium	k_{cat} (min^{-1})	K_{m1} (D-Ala) (μM)	K_{m2} (D-Ala) (μM)
S. typhimurium	644	1.9	540
E. coli Ddl A	444	5.7	550 (1200[b])
E. coli Ddl B	1018	3.3	1200 (1700[b])
E. faecium (VanA)	295	3400	38 000 (25 000[b])

[a]From [6,24].
[b]This work.

D-Ala (K_{m1}, Fig. 2), which is converted to the mixed anhydride by ATP, are typically 100–1000-fold lower than those for binding of the second, nucleophilic substrate (K_{m2}) [6]. The rate expression that describes this process is given in equation 2A (Fig. 2b) [8].

The disparate affinities of the two D-Ala sites complicate the analysis of inhibitor binding by the typical competitive methods, particularly for inhibitors that occupy both of these subsites. At concentrations of D-Ala low enough to permit competition between the inhibitor and D-Ala for the first site ([D-Ala] ≈ 1 μM), so little is bound to the second site that the reaction rate is very slow (~1/1000 of V_{max}); as a result, the initial, reversible binding step cannot be monitored under these conditions. At concentrations of D-Ala high enough to occupy both sites and give reasonable reaction rates ([D-Ala] ≈ 1 mM), however, inhibition is virtually overwhelmed by saturation of the first site.

For the dipeptidyl phosphinate and phosphonate inhibitors, the analysis is further complicated by a biphasic inactivation process; the degree of inhibition increases with time, presumably as the phosphinate (or phosphonate) is converted to the more tightly-bound, phosphorylated species, **P-1** [9–11]. The ultimate affinity of this or related compounds in the ternary complex with enzyme and ADP has not been determined accurately; recovery of activity is very slow and it is not known whether dissociation of the complex is truly a mirror of the association process. An overall inhibition constant, K_i^*, for dissociation of the **P-1** complex has been reported for the tightened complex of an *E. coli*

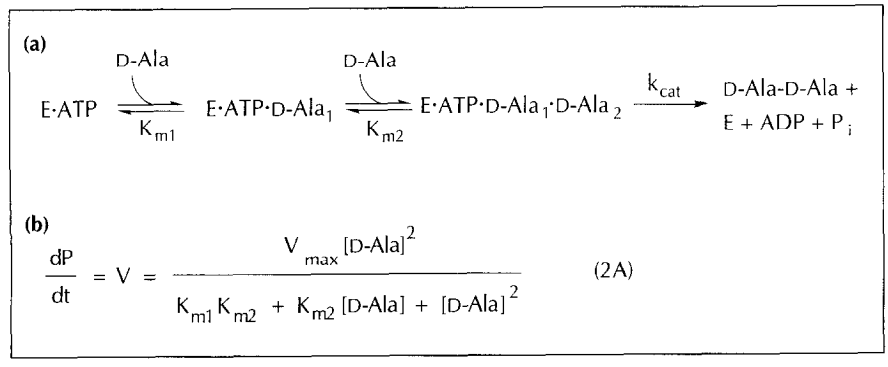

Fig. 2. Binding of substrate to the ligases occurs in two steps. **(a)** The stages of the reaction and **(b)** the overall rate expression reflecting the two-step substrate-binding process are shown.

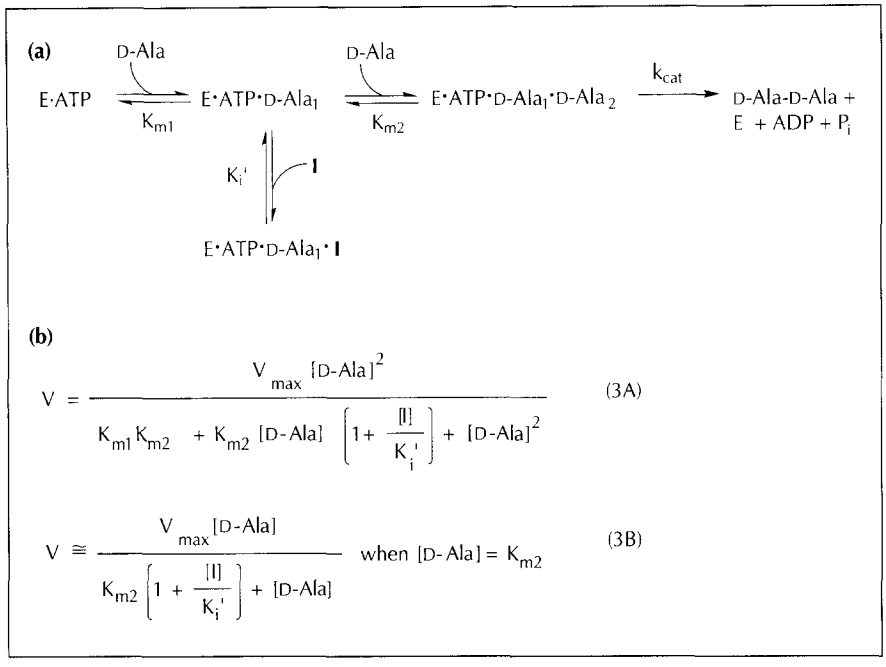

Fig. 3. Kinetic analysis of inhibitor (I) binding using the assumption that inhibitors bind to the ligase•D-Ala$_1$ complex. **(a)** The proposed reaction pathway and **(b)** the overall rate expressions that apply are shown.

ligase with the heptyl-substituted analog of compound **1** (with R = nC_7H_{15}), but this system apparently does not involve phosphoryl transfer to the inhibitor [4].

Results

Phosphinates **1a–c** and phosphonates **2a** and **2b** were evaluated as inhibitors for three ligase enzymes: the DdlA and DdlB enzymes from *E. coli* and the VanA mutant from *E. faecium*. We followed the lead of previous groups [4,8–10,12–15] and determined inhibitor affinity in the readily reversible phase of the inactivation process using a simple Dixon plot to calculate binding constants at [D-Ala] = K_{m2}. This analysis is derived from equation 3B (see Fig. 3b), which corresponds to inhibitor competition with the second D-Ala substrate as represented by the reaction mechanism in Figure 3. We therefore designate these constants as K_i' values in Table 2. Since the relevant binding event is that of the inhibitor to the free enzyme, not binding of the inhibitor to the enzyme-D-Ala$_1$ complex (see the discussion below), we also calculated inhibition constants using equation 4C (see Fig. 4b). This scheme represents competition with both D-Ala substrates according to the reaction mechanism in Figure 4; the K_i values derived in this manner are provided in Table 3.

In this study, we did not determine the time course of inhibitor phosphorylation, which is the second step in the inactivation mechanism, or measure the affinity of these final complexes. Preliminary evidence suggests, however, that the phosphonates come to equilibrium more rapidly than the phosphinates, reflecting either incomplete phosphorylation in the active site, or more rapid reversal of the phosphorylation step. This latter explanation is consistent with the differences observed for phosphinate and phosphonate inhibitors of glutamine synthetase, which also undergo phosphorylation in the enzyme active site [16,17].

Discussion

Mode of inhibition

The rapidly reversible step of inhibitor binding has been analyzed previously at [D-Ala] = K_{m2}, applying the Dixon analysis [18] to equation 3B [9]. The K_i' values of Table 2 were determined in this manner and thus can be compared directly to the previous results. Equation 3B is equivalent to equation 3A when [D-Ala] = $K_{m2} \approx 1000 \times K_{m1}$, since the additional term in the denominator, $K_{m1}K_{m2}$, is insignificant under these conditions. These expressions are valid for calculation of inhibition constants for binding of an inhibitor that is only in competition for the second D-Ala binding site, leading to formation of an enzyme•D-Ala1•inhibitor complex from enzyme•D-Ala1 (Fig. 3).

It is unlikely, however, that the dipeptidyl phosphinates and phosphonates bind exclusively or even primarily to the enzyme•D-Ala$_1$ complex. These inhibitors are bisubstrate analogs that are readily phosphorylated in the enzyme active site as alternative substrates to the tightly

Table 2. Reversible inhibition of ligases by phosphinate and phosphonate analogs: binding to ligase•D-Ala$_1$ complex.[a]

	DdlA[b]	DdlB[b]	VanA[b]
D-Ala[$PO_2^-CH_2$]D-Ala (**1a**)	27	4	60
D-Ala[$PO_2^-CH_2$]D-Leu (**1b**)	9	4	23
D-Ala[$PO_2^-CH_2$]D-Phe (**1c**)	3	3	11
D-Ala[PO_2^-O]D-Lac[c] (**2a**)	800	18	130
D-Ala[PO_2^-O]D-Hbut[c] (**2b**)	1000	20	100

[a] K_i' values in μM calculated according to equation 3B as described in [9].
[b] K_{m2} (DdlA) = 1.2 mM; K_{m2} (DdlB) = 1.7 mM; K_{m2} (VanA) = 25 mM.
[c] Lac = lactate, Hbut = α-hydroxybutyrate.

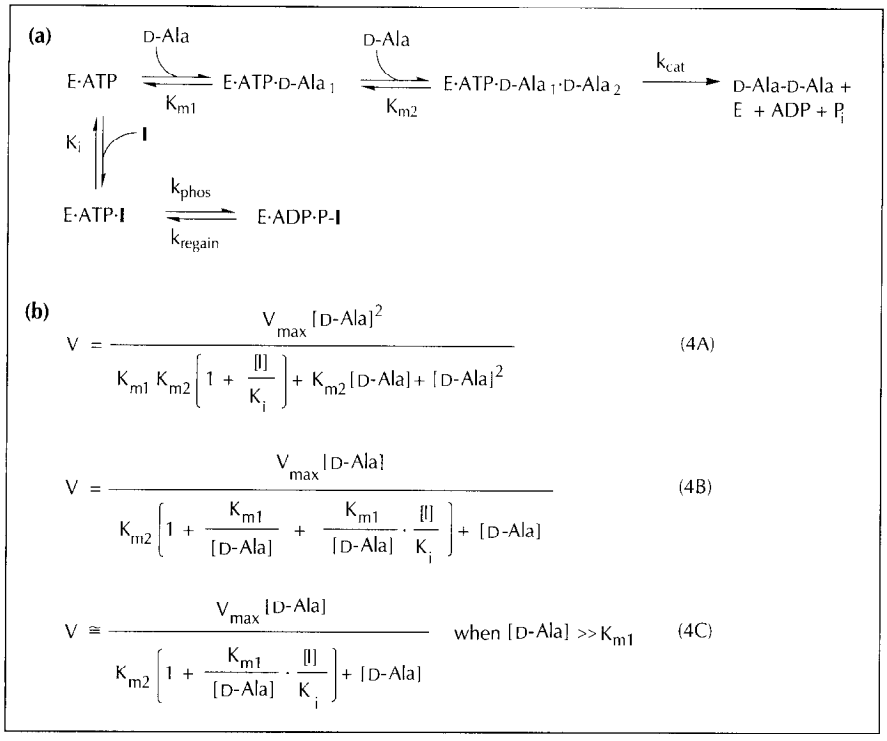

Fig. 4. Kinetic analysis of inhibitor (I) binding to the free enzyme. **(a)** The proposed reaction pathway and **(b)** overall rate expressions that apply are shown.

bound D-Ala; this phosphorylation logically requires the aminoethylphosphinyl moiety of the inhibitors to bind in the first D-Ala site, as indicated in Figure 4. Moreover, if the inhibitors were accepted as alternative ligands at the second D-Ala site, they would logically be ligated to D-Ala_1 and substrate turnover, rather than enzyme inhibition, would be expected. The most compelling evidence against the notion that these inhibitors bind to the ligase•D-Ala_1 complex is the fact that both the substrate specificity of the various ligases and the three-dimensional structure of the *E. coli* enzyme suggest that molecules as large as the dipeptidyl inhibitors cannot fit into the active site in the presence of D-Ala_1. We therefore conclude that these inhibitors probably compete with both D-Ala substrates for binding to the enzyme.

Inhibitor binding in competition with both D-Ala substrates, according to the scheme in Figure 4, is described by equation 4A (Fig. 4b). This expression can be rearranged to equation 4B, and simplified to the Michaelis–Menten relationship shown in equation 4C, recognizing that the ratio K_{m1}/[D-Ala] is small relative to 1 under the assay conditions, that is, when [D-Ala] = $K_{m2} \approx 1000 \times K_{m1}$. Thus the K_i' values determined from Dixon analysis of equation 3B must be corrected by the K_{m1}/[D-Ala] term of equation 4C to obtain the inhibition constants, K_i, for binding to free enzyme (Table 3). The two modes of analysis (Figs 3 and 4) only affect comparisons between the three enzymes; comparisons between inhibitors for the same enzyme are independent of the model used, since the factor K_{m1}/[D-Ala] drops out of a ratio of K_i values.

Table 3. Reversible inhibition of ligases by phosphinate and phosphonate analogs: binding to free enzyme.[a]

	DdlA[b]	DdlB[b]	VanA[b]
D-Ala[$PO_2^-CH_2$]D-Ala (**1a**)	55	3	4100
D-Ala[$PO_2^-CH_2$]D-Leu (**1b**)	18	3	1600
D-Ala[$PO_2^-CH_2$]D-Phe (**1c**)	6	2	750
D-Ala[PO_2^-O]D-Lac[c] (**2a**)	1600	12	8800
D-Ala[PO_2^-O]D-Hbut[c] (**2b**)	2000	13	6800

[a] K_i values in nM calculated according to equation 4C; K_{m1} (DdlA) = 6 μM; K_{m1} (DdlB) = 3 μM; K_{m1} (VanA) = 3 mM, taken from [5]
[b] K_{m2} (DdlA) = 1.2 mM; K_{m2} (DdlB) = 1.7 mM; K_{m2} (VanA) = 25 mM.
[c] Lac = lactate, Hbut = α-hydroxybutyrate.

Inhibitor specificity

The three ligases differ in their selectivities for the side chain of the carboxy-terminal moiety, as well as in their sensitivity to replacement of the methylene linkage of the phosphinates with the oxygen of the phosphonates. The oxygen was incorporated to provide a better mimic for the VanA mutant, which has been shown to catalyze ester formation from α-hydroxy acids in preference to amide formation from D-Ala [6,7]. (Comparison with the nitrogen-linked, dipeptidyl phosphonamidates is not possible, since these compounds are not stable at neutral pH [14].) It is interesting, therefore, that the phosphonates are weaker inhibitors than the phosphinates for all three ligases studied, although the least discrimination is seen with DdlB, not with the VanA mutant. There is little difference among the three enzymes in their selectivity for carboxy-terminal sidechains: all prefer the hydrophobic phenylalanyl and leucyl moieties over the alanine methyl group, although, again, the least selectivity is shown by DdlB. The major difference in substrate

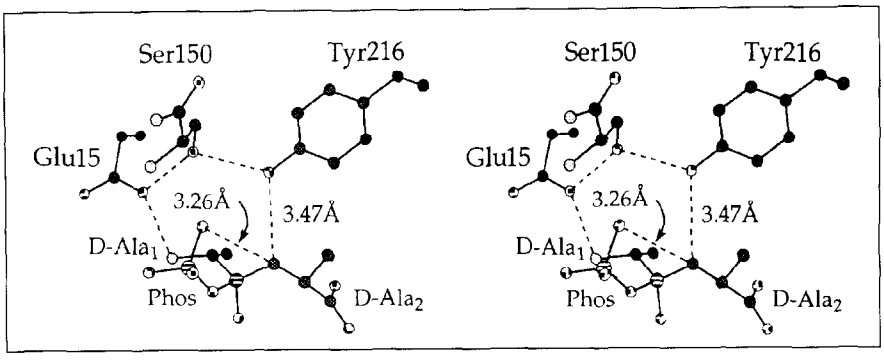

Fig. 5. A stereoview of the hydrogen-bonding network near **P-1a** in the DdlB active site. Potential hydrogen bonds are shown as dashed lines. Carbon atoms are black, oxygens white, nitrogens grey and phosphorus hatched.

selectivity between the wild-type ligases and VanA appears to be in the D-Ala_1 site.

The recently determined structure of the complex between the *E. coli* DdlB ligase and compound **P-1a**, the phosphorylated form of the D-Ala-D-Ala phosphinate **1a**, provides a starting point for structural analysis of the effects on inhibitor binding (Fig. 5) [3]. Although the inhibitor in this complex is already in the tight-binding form due to phosphorylation, the structure nonetheless provides an indication of the active-site residues that may interact with the linking atom (the nitrogen of the amide link in the case of dipeptide formation, or the oxygen of the ester link in depsipeptide formation). The amino-acid side chain of DdlB that is closest to the methylene linkage in compound **P-1a** is Tyr216, with an O–C distance of 3.5 Å. In the actual substrate complex (N in place of C), these atoms would be within hydrogen-bonding distance, and their interaction could be significant in substrate binding. Many of the active-site residues of DdlB are preserved in the DdlA enzyme and in the VanA mutant, with the significant exception of Tyr216, which is replaced by Lys in VanA. As Knox and coworkers point out [3], this mutation is probably responsible for the preference of the VanA enzyme for hydroxy acids over amino acids as the nucleophilic substrate.

Enzyme mechanism

One of the unresolved aspects of the acylation step in the ligase mechanism is how the proton(s) are transferred from the attacking nucleophile to the departing phosphate [3]. The initial proposal that the phenolic oxygen of Tyr216 is responsible has been ruled out by Shi and Walsh [11], who found that the Tyr216→Phe mutant of DdlB is almost as active catalytically as the native enzyme. They point out that a significant fraction of D-Ala is deprotonated in the pH range where the enzyme is active, and, moreover, that the difference in affinity at the two D-Ala binding sites can easily accommodate a mechanism in which D-Ala_1 binds as the zwitterion and D-Ala_2 binds as the anion.

There may not be any intermediary in the transfer of the proton from the nucleophile to the phosphate (Fig. 6a). If D-Ala_2 binds as the anion, proton transfer is not required until the tetrahedral intermediate tautomerizes prior to release of inorganic phosphate (Fig. 1). In the complex of DdlB with adduct **P-1a**, one of the phosphate oxygens is only 3.26 Å away from the methylene

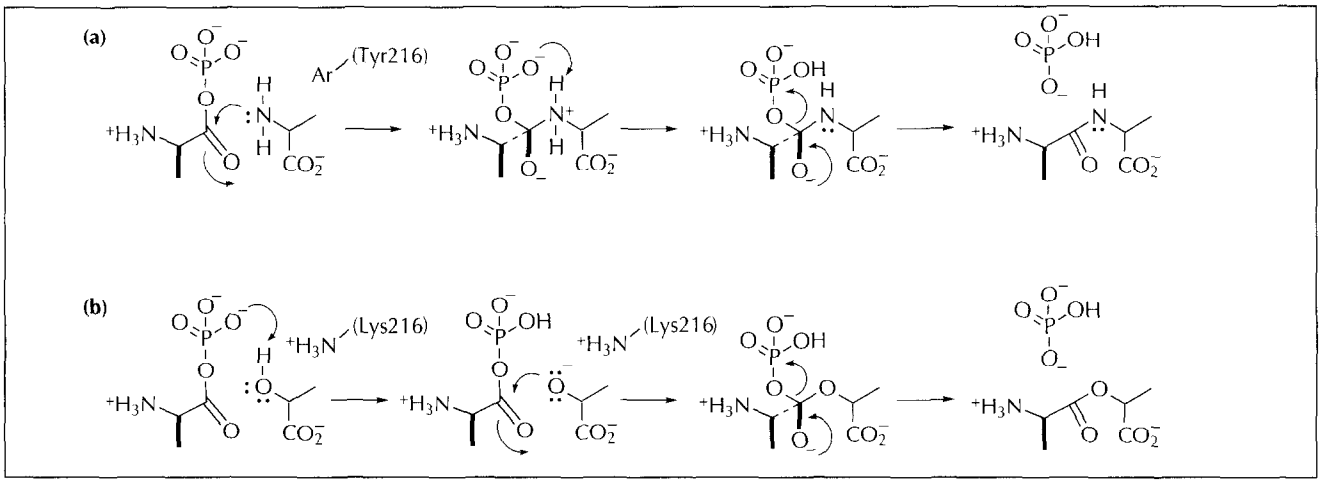

Fig. 6. Proposed mechanisms for the ligation reactions carried out by **(a)** wild-type ligases and **(b)** VanA. In both cases, D-Ala_1 binds to the first substrate-binding site as the zwitterion and becomes phosphorylated (see Fig. 1). In the case of the wild-type ligases (a), the second D-Ala binds as the anion and therefore does not need to be deprotonated prior to nucleophilic attack. After formation of the tetrahedral adduct, proton transfer from the nucleophilic nitrogen to the departing phosphate occurs directly, without involvement of the enzyme. In the case of VanA (b), the side chain closest to the nucleophile, Tyr216, is replaced with a Lys residue; the proximity of the cationic side chain of Lys216 acidifies the hydroxyl group of the α-hydroxy acid substrate, enabling the phosphate to deprotonate it and induce nucleophilic attack. No subsequent proton transfers are required to complete formation of the depsipeptide.

carbon that corresponds to the nitrogen of D-Ala$_2$ in the tetrahedral adduct, a distance that would permit proton transfer [3]. In the VanA mutant, the phosphate oxygen may also be the proton acceptor that activates the hydroxyl group of the α-hydroxy acid (Fig. 6b); the phosphate is nominally the strongest base in the vicinity, and it must pick up a proton to allow decomposition of the tetrahedral adduct. In this mechanism, Lys216 serves to acidify the hydroxyl group electrostatically, as previously suggested [3]. (See [19] for another case in which an enzyme induces the substrate phosphate group to serve as the catalytic base.)

Can we rationalize the modest differences in binding affinity between the phosphinate and phosphonate inhibitors in the light of the structural observations and the above mechanistic proposals? The ability to discriminate between the methylene- and oxygen-linked inhibitors is independent of the residue at position 216. The disparate enzymes DdlB and VanA show similar, four- to five-fold preferences for the phosphinates over the phosphonates, whereas DdlA and DdlB, which both have Tyr at position 216, differ significantly in their ability to bind the phosphonates. Thus, these differences appear to be unrelated to substrate preference and enzyme mechanism. Moreover, previous experience with phosphinate and phosphonate peptidase inhibitors has shown that differences in solvation between these inhibitors are at least as important in determining reversible binding affinities as their specific active site interactions [20].

Comparison between enzymes

The greatest difference between the three ligases is their absolute affinity for the inhibitors, as determined by the K_i values (Table 3). The drastically weaker binding of the inhibitors to the VanA mutant mirrors the reduced affinity of the enzyme for the substrates, especially at the D-Ala$_1$ site (Table 1). This reduction in inhibitor affinity for VanA is not as apparent from K_i' values (Table 2), since those analyses do not take into account competition with substrate binding at the D-Ala$_1$ site. As a starting point for mutation, the wild-type ligases have considerable affinity to sacrifice at the D-Ala$_1$ site, since the K_{m1} values of the enzymes for D-Ala are about 200–400-fold lower than the K_{m2} values. Indeed, the greatest change in substrate affinity for the VanA mutant has actually occurred at the D-Ala$_1$ site, even though altered specificity at the D-Ala$_2$ site, allowing hydroxy acids to function as substrates, is the ultimate result of the mutation. The large reduction in binding affinity to the VanA mutant for all the phosphorus-containing analogs has significant implications for the design of inhibitors specific for this enzyme.

Significance

D-Alanine:D-alanine ligase is an enzyme that is essential for bacterial cell wall synthesis. The subunit it produces is incorporated into a pentapeptide precursor of the peptidoglycan; this pentapeptide is the target of the antibiotic vancomycin. We have studied the specificity of inhibitor binding to wild-type (DdlA and DdlB) and to a mutant version (VanA) of this ligase. VanA, which is from a vancomycin-resistant bacterium, forms the D-Ala-D-hydroxybutyrate depsipeptide instead of D-Ala-D-Ala; incorporation of the depsipeptide unit in the growing cell wall leads to a modified peptidoglycan that is not complexed by vancomycin, thus allowing the host to escape the action of this antibiotic. Of the two series of inhibitors studied, the phosphinates are known to afford mimics of the tetrahedral intermediate of the wild type ligases; the phosphonates, in turn, were devised to mimic that for depsipeptide formation.

Inhibitors of this type have previously been analyzed as competitors with D-Ala, only for binding to the second substrate site in the rapidly reversible stage of inhibition. When evaluated as competitors for both D-Ala-binding sites, the phosphinates appear to bind the native DdlA and Ddlb enzymes three orders of magnitude more tightly than when assessed with the previous analysis. This difference is considerably less for the VanA mutant. Although VanA has altered substrate specificity at the second binding site, the major differences seen between this enzyme and the wild-type ligases are changes in substrate and inhibitor binding at the D-Ala$_1$ site. Interestingly, discrimination between phosphinate and phosphonate inhibitors does not parallel that between amino acid and hydroxy acid substrate selectivity.

A novel mechanism is proposed for the wild-type and mutant ligases, in which proton transfer from the attacking nucleophile to the departing phosphate occurs directly, without involvement of an enzymatic functional group. The fact that no enzymatic side chain is involved explicitly may explain why mutation from an N-acylase to an O-acylase is relatively straightforward, only requiring alteration of the electrostatic characteristics of the active site.

Materials and methods

Synthesis

Experimental procedures for preparation of compounds **1a-c** and **2a,b** and the details of their characterization are provided in the Supplementary material. A revised synthesis of the D-Ala-D-Ala phosphinate **1a** was developed and extended to the preparation of the D-Leu and D-Phe analogs **1b** and **1c** (Fig. 7). Phosphonous acid **3** was prepared and resolved by the reported procedure [21]. The D-enantiomer was converted to the trivalent tautomer by silylation, and added to the appropriate α-alkylacrylate esters to give the coupled phosphinates as mixtures of four diastereomers. The phosphonate derivatives were prepared from the same phosphonous acid using an *in situ* activation and

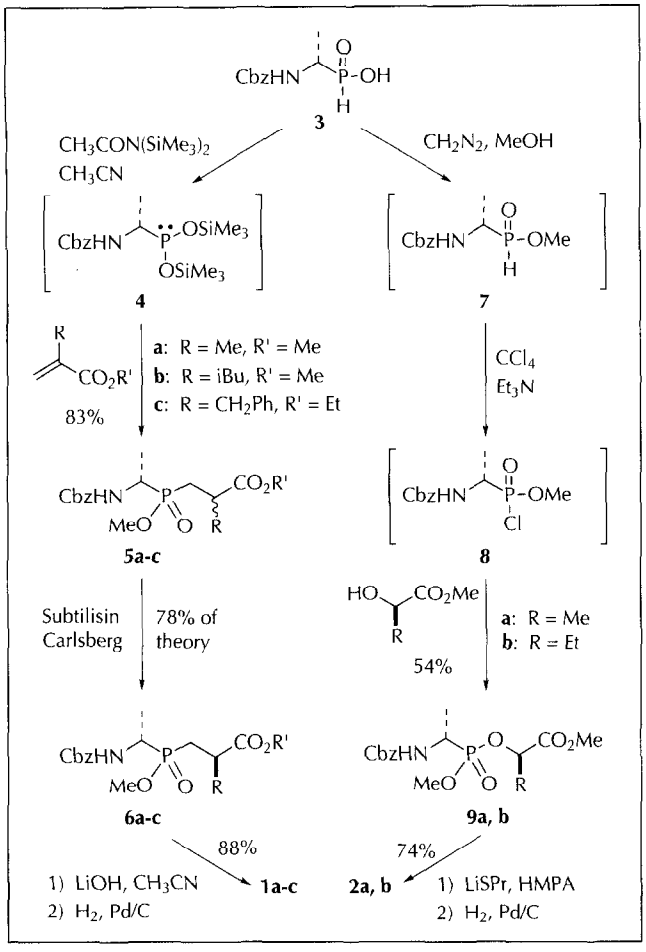

Fig. 7. Synthesis of inhibitors (see Materials and methods for details).

coupling protocol [22]. Diastereomeric phosphinates were resolved by digestion with the serine protease subtilisin Carlsberg; isomers with the L-configuration at the terminal center were hydrolyzed, allowing separation from the neutral, D,D-diastereomers. In the case of the phosphinates, simple alkaline hydrolysis and hydrogenolysis removed the esters and nitrogen protecting group. Selective cleavage of the phosphonate esters was achieved with lithium propanethiolate [23] prior to hydrogenolysis of the benzyl carbamate (Cbz) moiety. All of the inhibitors were obtained as the zwitterions as stable white solids.

Enzyme assays

The standard buffer used in all assays was 0.1 M Tris, 10 mM $MgCl_2$, and 10 mM KCl in doubly distilled water. The buffer was adjusted to pH 7.8 at room temperature and passed through a Millipore filter (0.45-μm pore size) prior to use. The buffer used in assays with *E. faecium* VanA was prepared in a similar manner except the pH was adjusted to 8.6 at room temperature. Assays were performed on a Uvikon 860 spectrophotometer at 37 °C, and data were analyzed with Kaleidagraph (Version 3.0.1, Adelbeck Software).

Determination of K_{m2} for the ligases

The assay solution included enzyme (~1.5 μg ml^{-1} for DdlA, ~0.5 μg ml^{-1} for DdlB, VanA concentration undetermined), 5 mM ATP, 50 μg ml^{-1} lactate dehydrogenase, 0.14 mg ml^{-1} pyruvate kinase, 5 mM NADH, 125 mM phosphoenolpyruvate (PEP), and D-Ala in a final volume of 1 ml. The concentration of D-Ala was varied from 0.25–4 x K_{m2}. The enzymatic rate was determined by monitoring the UV absorbance change at 340 nm to less than 10 % consumption of substrate, and the data was analyzed via non-linear regression according to equation 4A, using the K_{m1} values reported by Wright and Walsh [5]. Values for K_{m2} determined by non-linear regression do not differ significantly from those determined previously by Lineweaver–Burk analysis (Table 1) [24].

Inhibition of the ligases

The *E. coli* enzymes DdlA (~1.4 μg ml^{-1}) and DdlB (~0.5 μg ml^{-1}) and the *E. faecium* ligase VanA (~0.8 μg ml^{-1}) were assayed in the presence of 3 mM ATP, 10 μg ml^{-1} lactate dehydrogenase, 0.03 mg ml^{-1} pyruvate kinase, 5 mM NADH, 125 mM PEP, D-Ala at a concentration equal to K_{m2}, and the inhibitor in a total volume of 1 ml at 37 °C. The enzymatic rate was determined by monitoring the UV absorbance change at 340 nm.

Supplementary material available

Experimental details for the preparation of compounds **1a–c** and **2a,b**; kinetic plots for determination of K_i values.

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