D-Ala–D-X ligases: evaluation of D-alanyl phosphate intermediate by MIX, PIX and rapid quench studies

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Background: The D-alanyl–D-lactate (D-Ala–D-Lac) ligase is required for synthesis of altered peptidoglycan (PG) termini in the VanA phenotype of vancomycin-resistant enterococci (VRE), and the D-alanyl–D-serine (D-Ala–D-Ser) ligase is required for the VanC phenotype of VRE. Here we have compared these with the *Escherichia coli* D-Ala–D-Ala ligase DdlB for formation of the enzyme-bound D-alanyl phosphate, D-Ala_1-PO_3^{2-} (D-Ala_1-P), intermediate.

Results: The VanC2 ligase catalyzes a molecular isotope exchange (MIX) partial reaction, incorporating radioactivity from ^{14}C-D-Ser into D-Ala–^{14}C-D-Ser at a rate of 0.7 min^{-1}, which approaches kinetic competence for the reversible D-Ala_1-P formation from the back direction. A positional isotope exchange (PIX) study with the VanC2 and VanA ligases displayed a D-Ala_1-dependent bridge to nonbridge exchange of the oxygen-18 label of [γ-$^{18}O_4$]-ATP at rates of up to 0.6 min^{-1}; this exchange was completely suppressed by the addition of the second substrate D-Ser or D-Lac, respectively, as the D-Ala_1-P intermediate was swept in the forward direction. As a third criterion for formation of bound D-Ala_1-P, we conducted rapid quench studies to detect bursts of ADP formation in the first turnover of DdlB and VanA. With *E. coli* DdlB, there was a burst amplitude of ADP corresponding to 26–30% of the DdlB active sites, followed by the expected steady-state rate of 620–650 min^{-1}. For D-Ala–D-Lac and D-Ala–D-Ala synthesis by VanA, we measured a burst of 25–30% or 51% of active enzyme, respectively.

Conclusions: These three approaches support the rapid (more than 1000 min^{-1}), reversible formation of the enzyme intermediate D-Ala_1-P by members of the D-Ala–D-X (where X is Ala, Ser or Lac) ligase superfamily.

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Introduction

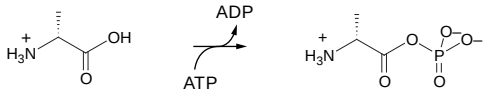
Clinically significant resistance to the antibiotic vancomycin in life-threatening infections by vancomycin-resistant enterococci (VRE) arises by reprogramming the peptidoglycan (PG) termini of the enterococcal cell wall. The normal D-Ala–D-Ala dipeptide termini, which are high-affinity sites for vancomycin binding, are replaced either by D-Ala–D-Lac depsipeptide termini (VanA and VanB phenotypes of resistance [1–7]) or by D-Ala–D-Ser dipeptide termini (VanC phenotype [8]). The D-Ala–D-Lac termini bind vancomycin three orders of magnitude less tightly [2], whereas the D-Ala–D-Ser moiety binds one order of magnitude less tightly than the D-Ala–D-Ala termini [9].

The reprogramming of the PG termini in the VRE phenotypes is caused by a switch from the sole production of the normal PG precursor D-Ala-D-Ala by the bacterial D-Ala–D-Ala ligase (Ddl) to production of D-Ala–D-X, where X is lactate in VanA and VanB and X is serine in the VanC phenotype. The D-Ala–D-X metabolites are generated by expression of either inducible (VanA and VanB) or constitutive (VanC) D-Ala–D-X ligases in competition with Ddl [3,10]. In the pathogenic VRE strains, the cytoplasmic D-Ala–D-Ala is selectively hydrolyzed by a VanX D-D-peptidase [4,11] while the D-Ala–D-X metabolites accumulate and become incorporated into the resistant PG termini.

A key molecular determinant of vancomycin resistance is thus the switch in specificity between Ddls, VanA and VanB ligases, and VanC ligases, from activation of D-Ala_2, as the nucleophilic partner in the D-Ala–D-X product-determining step, to selective activation of D-Lac (VanA and VanB) or D-Ser (VanC). For example, the VanA ligase has about a 30,000-fold enhanced selectivity for D-Lac over D-Ala_2 as compared with *Escherichia coli* DdlB [12], whereas the VanC2 ligase shows about a 240:1 preference in terms of catalytic efficiency for the incorporation of D-Ser over D-Ala_2 [13,14]. The structural basis of the differences in catalytic efficiency in the D-Ala–D-X ligases are now beginning to be unravelled, given X-ray structures of *E. coli* DdlB [15] and a Tyr216→Phe (Y219F) mutant [16], as well as very recent structures of a

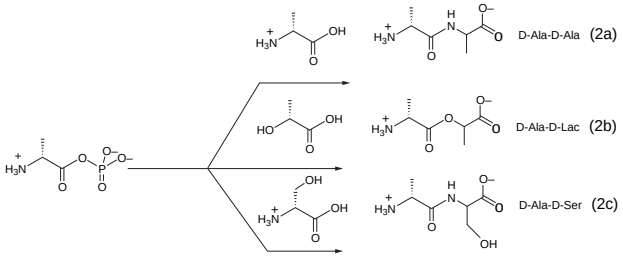
D-Ala–D-Lac ligase from the naturally vancomycin resistant *Leuconsotoc mesenteroides* [17] and a VanA ligase [18].

In terms of the catalytic mechanism, it is likely that all Ddl, VanA and B, and VanC forms of the D-Ala–D-X ligases use the common enzyme-bound intermediate D-Ala_1-PO_3 (D-Ala_1-P), which arises from attack of a D-Ala_1-carboxylate oxygen on bound ATP (equation 1).



(1)

The subsequent capture of D-Ala_1-P by the nucleophilic second substrate, D-Ala_2, D-Lac or D-Ser (equation 2a–c) leads to the distinct outcomes of the subfamilies of D-Ala–D-X ligases.

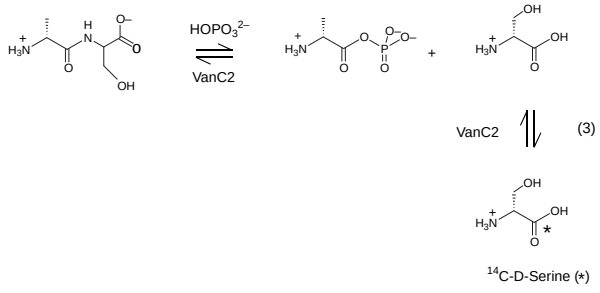


In previous studies of the *Salmonella typhimurium* D-Ala–D-Ala ligase (StDdl), we gained preliminary evidence for enzyme-bound D-Ala_1-P formation by using positional isotope exchange (PIX) and molecular isotope exchange (MIX) [19]. Here we extend experiments to both the *Enterococcus faecium* VanA (D-Ala–D-Lac ligase) and *Enterococcus casseliflavus* VanC2 (D-Ala–D-Ser ligase) and obtain evidence for the mixed D-Ala_1-P anhydride by PIX, MIX and rapid quench studies.

Results

VanC2 molecular isotope exchange (MIX) reaction

One approach to the detection of a tightly bound but reversibly formed intermediate during enzymatic catalysis is to see whether a partial reaction has occurred in the absence of overall catalysis. The proposed intermediate D-Ala_1-P, which is common to Ddl, VanA and VanC catalysis, should be formed in the forward direction in the absence of the nucleophilic co-substrate, D-Ala_2, D-Lac and D-Ser, respectively. Because there is still some residual affinity of the VanA and VanC ligases for D-Ala at subsite 2 [12,13], it is very difficult to prove that there is a partial exchange reaction and to suppress any small percentage flux of the overall forward reaction when D-Ala is present. Therefore, we used the VanC2 ligase and the back reaction to look for D-Ala_1-P during phosphorolysis of D-Ala-D-Ser by inorganic phosphate (Pi; equation 3).



First, we established that the overall back reaction, equation 4, could proceed in the presence of all components, D-Ala-D-Ser, Pi and ADP, using a coupled spectrophotometric assay to measure ATP production.

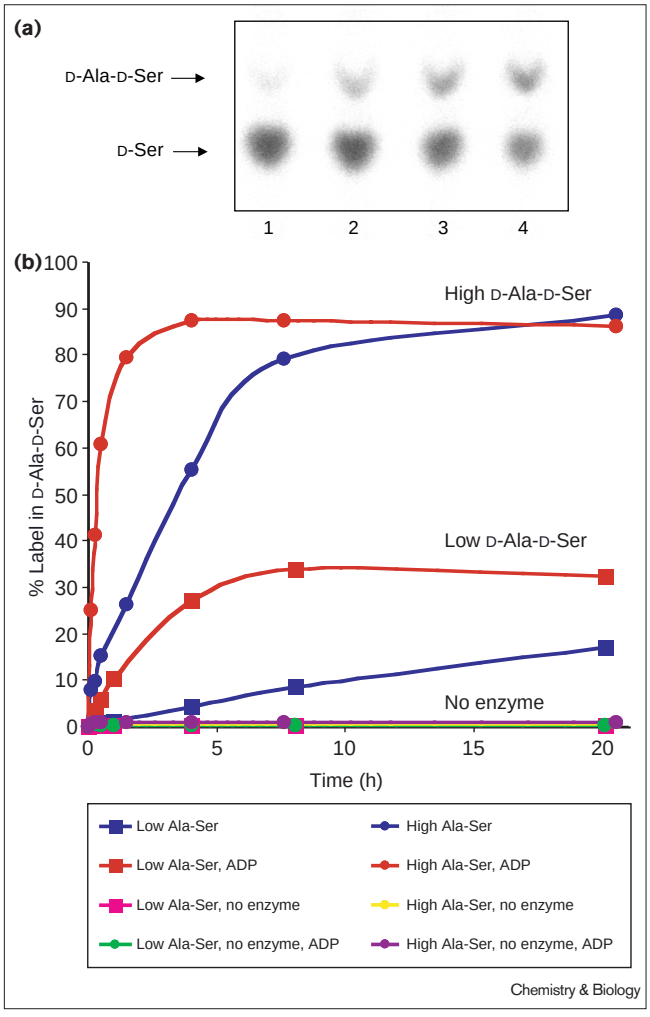
$$\text{D-Ala–D-Ser} + \text{Pi} + \text{ADP} \xrightarrow[\text{VanC2}]{} \text{D-Ala} + \text{D-Ser} + \text{ATP} \qquad (4)$$

At pH 7.5, the k_{cat} for VanC2 ligase production of ATP from D-Ala–D-Ser, Pi, and ADP was 1.7 min^{-1} at 25°C and 3.3 min^{-1} at 30°C. The K_m values for D-Ala–D-Ser (17 mM), Pi (23 mM) and ADP (88 μM) indicated a low affinity for both the D-,D-dipeptide and Pi substrates in the D-Ala–D-Ser phosphorolysis reaction. In comparison, the back reaction with D-Ala–D-Ala yielded a K_m of 300 mM, almost 20-fold higher than the value for D-Ala–D-Ser.

At this point, we assessed the MIX partial reaction using VanC2 ligase, D-Ala–D-Ser and Pi, but not ADP, so that any enzyme-bound D-Ala_1-P formed by D-,D-dipeptide phosphorolysis (equation 3) could not be captured in the forward direction, as ADP was absent, and could only partition backwards through capture by D-Ser. If ^{14}C-D-Ser is included in such partial incubations, and if it can compete with D-Ser released in the initial phosphorolysis step, then the resynthesized D-D-dipeptide will be radioactive, D-Ala-^{14}C-D-Ser. Several cycles of catalytic phosphorolysis of D-Ala–D-Ser and resynthesis of D-Ala-^{14}C-D-Ser will lead to macroscopic exchange of radioactivity from the added monomer ^{14}C-D-Ser into the dipeptide D-Ala–^{14}C-D-Ser.

As shown in Figure 1a, radio thin layer chromatography (TLC) analysis of such partial exchange incubations shows time-dependent accumulation of radioactive D-Ala–D-Ser. The exchange of label into dipeptide is, as anticipated, dependent on the VanC2 ligase, Pi and D-Ala–D-Ser. Figure 1b shows rates of exchange at both low (1.5 mM) and high (150 mM) levels of D-Ala–D-Ser. Because the K_m of D-Ala-D-Ser in the overall back reaction is 17 mM (see above), the 1.5 mM dipeptide concentration is well below saturation, whereas the 150 mM concentration is saturating. We held the concentration of ^{14}C-D-Ser at 0.6 mM because of specific radioactivity limitations

Figure 1



MIX analysis of reversible intermediate formation from the back direction. **(a)** Representative thin layer chromatogram showing the separation of [^{14}C]-D-Ala–D-Ser from [^{14}C]-D-Ser for MIX experiments. For lanes 1 to 4, 1.5 mM D-Ala-D-Ser, 0.6 mM [^{14}C]-D-Ser, 20 mM KH_2PO_4, 10 mM $MgCl_2$,10 mM KCl, 100 mM HEPES, pH 7.5, and 10 μM VanC2 were incubated at room temperature for 1, 4, 8 or 20 h, respectively. **(b)** MIX by VanC2 at low (1.5 mM) and high (150 mM) D-Ala–D-Ser concentrations under the conditions described in (a) with or without addition of 18 mM ADP. Reaction mixtures were separated by TLC and quantified as described in the Materials and methods section.

(commercial material available at 55 mCi/mmol): addition of nonradioactive D-Ser lowered the signal-to-noise ratio to unacceptable levels. Illustrating the effect of adding the missing co-substrate ADP and allowing the overall back reaction to proceed and thus a net backward/forward equilibrium to be established, the red lines of Figure 1b show that the addition of saturating concentrations of ADP speeds up the MIX exchange kinetics 3- to 10-fold. It was possible to vary the Pi concentration in the MIX experiments and determine a K_m of 5 mM (compared with 23 mM in the overall back reaction).

Table 1

Molecular isotope exchange by VanC2 increases with ADP and high D-Ala–D-Ser.

ADP (mM)	0	18	0	18	0	18
D-Ala–D-Ser (mM)	1.5	1.5	100	100	150	150
v* (min^{-1})	0.007	0.10	0.36	1.1	0.68	2.3

*Reaction conditions as in Figure 1.

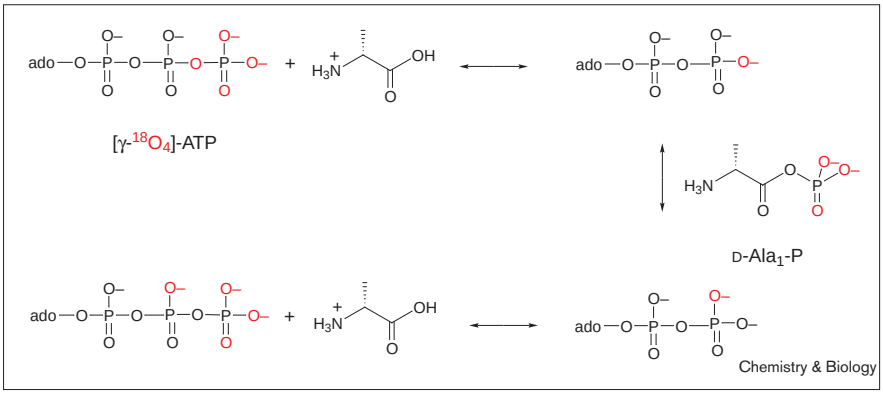
Table 1 shows that the MIX rate at saturating levels of D-Ala–D-Ser and Pi, and 0.6 mM ^{14}C-D-Ser was 0.68 min^{-1} in the absence of ADP and 2.3 min^{-1} when saturating concentrations of ADP were present. The 0.68 min^{-1} data measured using 0.6 mM ^{14}C-D-Ser were collected under sub-saturating conditions (D-Ser K_m = 2.6 mM for forward reaction [14]) and thus extrapolation to saturating D-Ser could possibly increase the rate to a kinetically competent level. These results confirm the ability of the VanC2 ligase, in the absence of ADP and therefore in the absence of a complete back reaction, to catalyze the Pi-mediated phosphorolysis of D-Ala–D-Ser to form an enzyme-bound intermediate that is reversibly reactive with ^{14}C-D-Ser from solution. D-Ala$_1$-P fits these requirements: its formation/capture rate approaches kinetic competency for the flux through the overall reaction.

VanA and VanC2 positional isotope exchange (PIX)

To test for D-Ala$_1$-P formation in the forward direction during D-Ala–D-X ligase action, and thus complement our back-direction results from MIX, we next used a non-radioactive isotope exchange technique known as positional isotope exchange (PIX) [20], which has proved useful for many enzymes that use ATP to establish reversible cleavage of the $^{\beta}$P–O–$^{\gamma}$P bond [21]. Starting with [γ-$^{18}O_4$]-ATP, incubations were conducted with either enzyme, with or without D-Ala, and with or without the specific co-substrates D-Lac (VanA) or D-Ser (VanC2). Reversible cleavage of ATP to ADP and D-Ala$_1$-P and reformation of ATP were detectable by a net exchange of the [β,γ-]-bridge oxygen-18 with the oxygen-16 that had been in the non-bridge β-position in the starting [γ-$^{18}O_4$]-ATP, which we measured by ^{31}P-NMR (Figure 2) [22]. In the absence of D-Ala, no PIX was detected for either the VanC2 or the VanA ligase, arguing against the formation of an E-X-PO_3^{-2} intermediate prior to D-Ala$_1$-P formation; nor was there any PIX detectable in the absence of either ligase.

When VanA (5 μM) was incubated with 2 mM [γ-$^{18}O_4$]-ATP and 1 mM D-Ala for 8 h at pH 6.0 and 30°C, about 10% of the oxygen-18 label at the [β,γ]-bridge position became scrambled with oxygen-16 label from the β-non-bridge position. We chose a pH of 6 to minimize capture by D-Ala$_2$ in the forward direction, as the K_m for D-Ala$_2$ at this pH was >> 200 mM [23,24]. Under these conditions, there was no net hydrolysis of ATP detectable above the

Figure 2



D-Ala dependent, positional isotope exchange reaction (adapted from [19]). The [β,γ]-bridge oxygen-18 is scrambled with the β-nonbridge oxygen-16 of [γ-$^{18}O_4$]-ATP.

0.1 mM limit of detection (Table 2). To determine the rate of $^{18}O/^{16}O$ scrambling, v_{ex}, by VanA, we used the following equation:

$$v_{ex} = \frac{-(A_o)}{t} \times [\ln(1-F)] \quad (5)$$

where F is the fraction of equilibrium attained at time *t* and A_o is the concentration of the original nucleotide pool [25] giving a PIX rate of 0.14 min^{-1} at 1 mM D-Ala. Because this concentration was below the K_m for D-Ala_1 (K_m = 3.2 mM at pH 6.0), the PIX incubations were repeated at 5 mM D-Ala, which produced a PIX exchange rate of 0.37 min^{-1}. This PIX rate was calculated using equation 6, where X corrects for the fraction of change in the original nucleotide pool [25], because there was a slow detectable ATPase rate of 0.15 min^{-1} using 5 mM D-Ala.

$$v_{ex} = \frac{X}{\ln(1-X)} \times \frac{(A_o)}{t} \times [\ln(1-F)] \quad (6)$$

Over the 8 h incubation, this 0.37 min^{-1} PIX rate corresponds to about 180 molecules of ATP turned over for nonbridge/bridge exchange for each VanA ligase molecule, emphasizing the catalytic nature of the process. It is not clear whether the slow net ATPase activity at 5 mM D-Ala is the result of a slow production of a small amount of D-Ala–D-Ala from D-Ala binding at subsite *2* or whether it reflects adventitious release and hydrolysis of enzyme-bound D-Ala_1-P. We then set up a reaction mixture with VanA ligase, 1 mM D-Ala and 10 mM cosubstrate D-Lac $15 \times K_m$ [23] until 50% of the starting *2* mM [γ-$^{18}O_4$]-ATP was consumed (4.5 h). No PIX was detected above the threshold limit of 0.01 min^{-1}. Thus, the D-Lac cosubstrate suppressed the PIX by at least a factor of 37 (0.37 min^{-1} to less than 0.01 min^{-1}), as it promoted the net flux in the forward direction (k_{cat} for D-Ala–D-Lac formation = 34 min^{-1}).

Analysis of the D-Ala-D-Ser-forming VanC2 ligase by PIX yielded similar results, consistent with reversible cleavage of ATP in the presence of D-Ala_1 to ADP and D-Ala_1-P in the ligase active site, followed by rotation of the torsio-symmetric $^{\beta}PO_3$ and religation to give ATP with a net $^{\beta}$P-O-$^{\gamma}$P nonbridge/bridge scrambling. Table 2 shows that 1.2 μM VanC2 ligase and 0.5 mM D-Ala (K_{m1} = 1.6 mM, K_{m2} >> 100 mM [14]), in the presence of *2* mM [γ-$^{18}O_4$]-ATP, yielded a PIX rate of 0.33 min^{-1}, without net ATPase activity. Raising the D-Ala concentration fourfold to *2* mM increased the PIX rate to 0.57 min^{-1}, but a net ATPase activity of 0.30 min^{-1} became detectable. In

Table 2

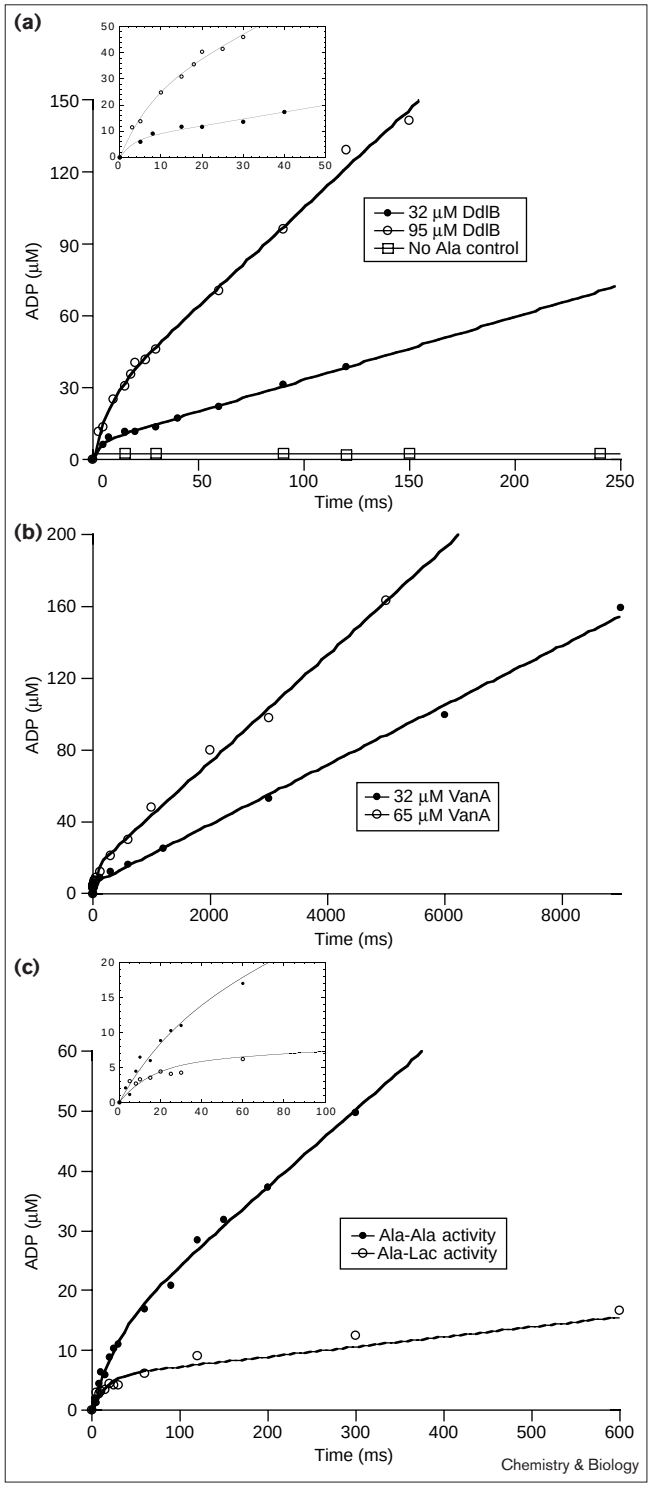
PIX results for VanA and VanC2.

Enzyme	(μM)	D-Ala (mM)	D-Lac or D-Ser (mM)	ADP formed (mM)	ATPase rate (min^{-1})	PIX rate (min^{-1})
VanA	5	1	0	<0.1	<0.04	0.14
VanA	5	5	0	0.36	0.15	0.37
VanA	0.4	1	10	0.84	8.8	<0.01
VanC2	3.4	0.5	0	<0.1	<0.06	0.33
VanC2	3.4	2	0	0.51	0.30	0.57
VanC2	0.4	2	20	0.98	10	<0.01

Reaction conditions as in the Materials and methods section.

Figure 3

Rapid quench analysis of ADP burst in the forward direction. **(a)** Effect of enzyme concentration on the burst amplitude and linear rate of D-Ala–D-Ala synthesis by DdlB. Assays contained 250 μM ATP, DdlB, 30 mM D-Ala, 10 mM $MgCl_2$, 10 mM KCl, and 100 mM HEPES, pH 7.8, at 30°C and were quenched with EDTA. Using 32 μM total DdlB, the burst rate is 18,000 min^{-1}, the linear rate is 620 min^{-1} (compared to the the steady-state rate of 658 min^{-1} from the lactate dehydrogenase/pyruvate kinase coupled assay), and the burst amplitude is 26% of the active enzyme concentration. The reaction with 95 μM total DdlB has a burst rate of 7900 min^{-1}, a linear rate of 650 min^{-1} (steady state rate of 658 min^{-1}), and a burst amplitude of 30% of the active DdlB concentration. **(b)** D-Ala–D-Lac synthesis by VanA. Assays contained VanA, 500 μM ATP, 10 mM $MgCl_2$, 10 mM KCl, amino acids and 100 mM MES, pH 6.2, at 30°C and were quenched with EDTA. Open circles, 65 μM VanA, 40 mM D-Ala, and 40 mM D-Lac; filled circles 32 μM VanA, 30 mM D-Ala and 30 mM D-Lac. Using 32 μM total VanA, the burst rate is 3,780 min^{-1}, the linear rate is 44 min^{-1} (steady-state rate of 43 min^{-1}), and the burst amplitude is 25% of the active enzyme concentration. The reaction with 95 μM total VanA has a burst rate of 1100 min^{-1}, a linear rate of 39 min^{-1} (steady-state rate of 40 min^{-1}), and a burst amplitude of 30% of the active VanA concentration. **(c)** Comparison of D-Ala–D-Ala and D-Ala–D-Lac synthesis by VanA. D-Ala-D-Lac synthesis as in (b) using 32 μM VanA. For D-Ala–D-Ala synthesis, 32 μM VanA was reacted with 500 μM ATP, 10 mM $MgCl_2$, 10 mM KCl, 200 mM D-Ala, and 100 mM TRIS, pH 8.4, at 30°C and then quenched with EDTA. For D-Ala–D-Ala synthesis the burst rate is 2100 min^{-1}, the linear rate is 341 min^{-1} (steady-state rate of 344 min^{-1}), and the burst amplitude is 51% of the active VanA concentration. The burst rate, linear rate and burst amplitude were determined on the basis of the concentration of active DdlB (80% of total enzyme) for (a) and active VanA (71% of total enzyme) for (b) and (c) using equation 8.



analogy to the VanA ligase, there is a D-Ala concentration range for VanC2 ligase that promotes PIX without net flux. When the co-substrate D-Ser was added at 20 mM ($8 \times K_m$ [14]) and the reaction with VanC2 was followed to consumption of 50% of the [γ-$^{18}O_4$]-ATP (4.5 h), again there was total suppression of the PIX (less than 0.01 min^{-1}), consistent with at least a 60-fold (from 0.6 min^{-1} to 0.01min^{-1}) partition of the D-Ala_1-P intermediate in the forward direction to make the D-Ala–D-Ser product. The net k_{cat} of 210 min^{-1} for D-Ala–D-Ser formation agrees with this anticipated partition [14].

DdlB and VanA rapid quench studies

To assess whether enzyme-bound intermediates or products accumulated during a catalytic cycle, we carried out rapid quench studies to analyze the kinetics during both the first turnover and then subsequent turnover cycles. This approach requires large amounts of enzyme and seeks to detect amounts of product up to concentrations that are stoichiometric with the amount of enzyme present. We chose to look for ADP formation as a measure of enzymatic cleavage of substrate [α-^{32}P]-ATP, because ADP would be stable under EDTA or acid quench conditions. The [α-^{32}P]-ADP product was readily and quantitatively separable from the ATP substrate by TLC [26]. Two constraints were (1) the requirement for high concentrations of ligase, satisfied by *E. coli* DdlB and *E. faecium* VanA but not the poorly soluble *E. casseliflavus* VanC2 ligase, and (2) a low K_m for ATP, allowing burst studies at saturating [α-^{32}P]ATP, again satisfied for DdlB and VanA but not VanC2 [12,13,27].

When *E. coli* DdlB at concentrations of either 32 μM or 95 μM was mixed with 250 μM [α-^{32}P]-ATP ($2.1 \times K_m$)

and 30 mM D-Ala ($3000 \times K_{m1}$, $30 \times K_{m2}$ [27]), a burst of [α-^{32}P]-ADP formation was clearly detectable after EDTA (Figure 3a) or acid quench (data not shown). There was negligible amount of radioactive ADP produced in the absence of D-Ala and in the absence of DdlB. Extrapolation of the amount of ADP produced back to the *y*-axis using equation 8 gave equivalent amounts relative to total enzyme concentration for both the 32 μM and 95 μM ligase concentrations [28]. After correcting for the fraction of active ligase (about 80% by titration with the transition-state analog phosphinophosphate [29]), the ADP burst values are 26–30% of the amount of DdlB, a substantial fractional accumulation. The subsequent rates of turnover after the burst are calculated as 620–650 min^{-1}, in agreement with the k_{cat} determined from steady-state kinetic experiments. The extrapolation of the burst rates has substantial error because of the small signal and short time period but gives a range of 7900–18,000 min^{-1}. Using the burst and linear rates from the curve fit of the time courses in Figure 3a and equations 7–10 [28], we calculated a value of 7000–18,000 min^{-1} for the rate of substrate binding and formation of ADP on the enzyme active site (k_1) and a value of 640–710 min^{-1} for the remaining steps in catalysis and product release (k_2). On the basis of these k_1 and k_2 rate constants, the anticipated burst magnitude is 38–43% of the amount of active enzyme (equation 11) [30], compared with the 26–30% observed. The observation of lower burst rates than expected might reflect an internal equilibrium of D-Ala$_1$-P to D-Ala$_1$ and ATP in the active site of this and other D-Ala-D-X ligases.

$$\mathrm{E} + 2\text{D-Ala} + \mathrm{ATP} \xrightarrow{k_1} \mathrm{E} - [\text{D-Ala}_1 - \mathrm{P} + \text{D-Ala} + \mathrm{ADP}] \xrightarrow{k_2} \mathrm{E} + \text{D-Ala} - \text{D-Ala} + \mathrm{ADP} + \mathrm{Pi} \quad (7)$$

$$Y = Ae^{-Bt} + L[E]t \quad (8)$$

where burst rate:

$$B = k_1 + k_2 \quad (9)$$

and linear rate:

$$L = \frac{k_1 k_2}{k_1 + k_2} \quad (10)$$

and burst magnitude:

$$A = [E]\left(\frac{k_1}{k_1 + k_2}\right)^2 \left(\frac{1}{1 + K_m/[S]}\right)^2 \quad (11)$$

The key issue is that a burst of ADP suggests that some subsequent step, such as capture of D-Ala$_1$-P or product release, limits turnover. We would expect that ADP formation is concomitant with D-Ala$_1$-P formation, however, the subsequent rate of capture by D-Ala$_2$ in the enzyme active site versus the rate of product release is not specifically addressed in these burst studies.

The VanA ligase at pH 6 is much less able to make D-Ala–D-Ala, preferentially directing flux to D-Ala–D-Lac. We therefore used rapid quench studies at this pH and 30°C with 32 μM VanA, 500 μM [α-^{32}P]-ATP ($4.3 \times K_m$), 30 mM D-Ala ($9 \times K_{m1}$, $<< K_{m2}$) and 30 mM D-Lac ($43 \times K_m$) (Figure 3b) [12,23]. A burst of [α-^{32}P]-ADP in the range of 25 to 30% of the active sites of VanA was detected using an EDTA quench. The subsequent turnover rate of 44 min^{-1} (based on the active enzyme concentration of 71% of the total enzyme) correlates with the independently measured steady-state k_{cat} value of 43 min^{-1}. A doubling of enzyme to 65 μM VanA doubled the amount of the burst of ADP. On the basis of an estimation of k_1/k_2 ratios and the ATP concentration at $4.3 \times K_m$, we calculated an expected burst magnitude of 61–64% of the active sites, indicating the observed burst amplitude was about half the anticipated amplitude. The VanA rapid quench studies were repeated at pH 8.4 (Figure 3c), where the D-Ala$_2$ drops from > 200 mM to a saturable 19 mM [23] and k_{cat} for the D-Ala–D-Ala flux increases to 341 min^{-1}. Rapid quench reactions under these conditions confirms a turnover rate of 344 min^{-1}, and there was again a burst of ADP, corresponding to 51% of the active VanA present. Even with a sevenfold faster k_{cat}, there is still a burst of enzyme-bound ADP formation.

Discussion

The various subfamilies of the D-Ala–D-X ligases, which provide D-,D-dipeptides or D-,D-depsipeptides (D-Ala–D-Lac) for bacterial peptidoglycan synthesis, are members of a superfamily of proteins with a fold called the 'ATP grasp' fold [31–34]. Most of these enzymes couple carboxylate and amine substrates to create an amide linkage in a ligated product as ATP is cleaved to ADP and Pi [31]. The common thermodynamic role is to drive the accumulation of the amide product by activation of the carboxylate partner for capture by the amine cosubstrate. The common mechanistic role for ATP in this architectural class of enzymes is likely to be attack on the electrophilic γ-PO$_3$ group by the substrate carboxylate to generate a mixed acyl phosphoric anhydride held in the active site and activated for amide formation and C–OPO$_3^{2-}$ bond cleavage to release the original γ-phosphate of ATP as inorganic phosphate [20,35,36]. In the specific context of the D-Ala–D-X ligases, for example DdlB, VanA and VanC2, the acyl phosphate would be D-Ala$_1$-P (equation 1).

It is expected that such intermediates in this C–N ligase enzyme superfamily remain tightly bound, as they would be hydrolytically labile if released, and in previous studies on all three D-Ala–D-X ligases, there was no indication of

uncoupling of ATP cleavage from amide product formation [23]. To understand how the different D-Ala–D-X ligases control the reaction flux to distinct co-substrates, D-Ala_2 (DdlB), D-Lac (VanA) or D-Ser (VanC2; equation 2a–c), we have undertaken to validate the hypothesis that D-Ala_1-P is a common intermediate.

In the absence of information about whether an enzyme-bound intermediate, such as D-Ala_1-P, accumulates to substantial substoichiometric concentration in the enzyme population, an initial sensitive test is the detection of its reversible formation using radioactive exchange. A classical approach is to look for a partial reaction occurring in the absence of one of the co-substrates, which is interpreted as existence of the intermediate. For example, the typical assay for aminoacyl tRNA synthetases, which is the amino acid-dependent exchange of ^{32}P radioactivity from ^{32}P-pyrophosphate (PPi) into ATP, monitors the production of the aminoacyl-AMP intermediate and PPi from the reaction of ATP with the amino acid (aa), in the absence of the tRNA co-substrate [37]. Whereas the aa-AMP remains tightly bound, the PPi dissociates and mixes in solution with ^{32}P–PPi, and when ^{32}P–PPi rebinds and recaptures the aminoacyl-AMP, the nascent ^{32}P-ATP that falls off is radiolabeled. Repetition for many catalytic cycles moves the ^{32}P from PPi into ATP. Thus, even if the aminoacyl-AMP stays tightly bound and does not accumulate in a large fraction of the enzyme molecules, its reversible formation can be detected. Finally, because the co-substrate tRNA is omitted, the movement of ^{32}P from PPi into ATP cannot arise from a contaminating flux of the overall forward and backward reaction; the isotope exchange under partial reaction conditions is mechanistically diagnostic of aa-AMP formation in the absence of the co-substrate tRNA.

The analogous partial reaction of radioactive exchange in the forward direction of D-Ala–D-Ala and other D-Ala–D-X ligases would be a ^{32}Pi exchange into ATP, dependent on D-Ala. This exchange is detectable, but because D-Ala is both substrate 1 and substrate 2, one cannot rule out exchange from the complete reaction going forward and backwards. The same is also true for VanA and VanC2 ligases — even though D-Ala_2 is much poorer as a substrate than D-Lac or D-Ser, respectively [12,13]. Thus, the radioactive exchange dependent on D-Ala does not unambiguously prove the partial reaction.

On the other hand, the back reaction should also yield D-Ala_1-P as a reaction intermediate, and whereas DdlB is unsuitable because D-Ala–D-Ala is the substrate in the back direction, and VanA is less suitable because of the hydrolytic lability of the D-Ala–D-Lac depsipeptide substrate, the VanC2 ligase with D-Ala–D-Ser offers the possibility that a radioactive molecular isotope exchange of ^{14}C-D-Ser into the D-Ala–D-Ser dipeptide, dependent on Pi but independent of ADP, might be diagnostic for D-Ala_1-P reversible formation. Indeed, the Pi-mediated phosphorolysis of D-Ala–D-Ser does proceed as an independent partial reaction. The equation of kinetic competence of reversible formation of D-Ala_1-P in the partial reaction compared with the overall back reaction was assessed by comparison with the overall k_{cat} for D-Ala-D-Ser + Pi + ADP conversion to ATP. The partial reaction is about threefold slower than the overall reaction, which might be due to a subsaturating concentration of D-Ser. There is reason to believe that the VanC2 and VanA ligases have loops that close over the D-Ala_1-P and ADP during catalysis, and so the absence of ADP could be a significant deterrent to full catalytic efficiency [15,18]; Finally, one of the potential kinetic limitations to MIX radioactivity transfers is the requirement for the release of the product equilibrates with the radioactive isotope, here the D-Ser. If its release or its rebinding (as ^{14}C-D-Ser) is anomalously slow (in the absence of ADP) this could limit apparent kinetic competency.

Because MIX radioactivity studies require the dissociation and rebinding of substrates in a partial reaction, the PIX technique was invented for ATP-cleaving enzymes so that bound products need not be released and mix with molecules in solution [20]**.** In the version of PIX that we used here, with regiospecifically prepared [γ-$^{18}O_4$]-ATP, cleavage in the enzyme active site to yield ADP (and in this case D-Ala_1-P) can be monitored if the β-phosphoryl group of ADP is free to rotate. Given the torsiosymmetry of the β-PO_3 group, its religation after free rotation would yield ATP with two out of three ATPs containing ^{16}O rather than the initial ^{18}O in the [β,γ]-bridge position (Figure 2). Thus, PIX is a more subtle probe for the reversible cleavage of ATP and not subject to the vagaries of kinetically slow release of co-products, although there is still the requirement for the β-^{16}O-γ-ATP to be released and replaced with starting β-^{18}O-γ-ATP many times to observe PIX on the bulk ATP. Indeed, both VanA and VanC2 ligases show PIX reactions that are dependent on D-Ala under conditions where binding to the second subsite is unlikely. The PIX probe is successful for the forward direction to the extent that D-Ala-dependent cleavage of ATP monitors D-Ala_1-P formation. When the co-substrate D-Lac (VanA) or D-Ser (VanC2) is added, all the D-Ala_1-P is captured and swept through to product, suppressing the PIX because ATP reformation and release does not occur. The PIX studies show that 200–400 molecules of ATP are reversibly cleaved and ADP rotated in the active site per molecule of VanA or VanC2 ligase, so the inferred D-Ala_1-P formation is reversible in the absence of nucleophilic cosubstrate. We will return to the issue of kinetic competence after discussing the rapid quench results.

Our third approach to detection of enzyme-bound species during turnover was rapid quench to quantitate whether the ADP product is formed more rapidly in the first turnover than the rate of the overall steady-state (k_{cat}). In

these studies, the labeled species was [α-^{32}P]-ATP and the product evaluated was the corresponding [α-^{32}P]-ADP. There was good evidence for burst kinetics in the first turnover of DdlB and VanA ligases. The VanC2 ligase was not evaluated owing to low solubility. The rates of subsequent turnovers correlated with steady-state k_{cat} values for DdlB and VanA ligases, supporting the first turnover results. As the burst measures ADP, the identity of the other species on the enzyme is not directly addressed. Whereas for DdlB it could either be the D-Ala$_1$-P or the D-Ala–D-Ala dipeptide product, yet to be released, for VanA ligase at pH 6 it is most likely that the ADP formation rate and stoichiometry in single turnover are a surrogate for equal amounts of D-Ala$_1$-P in the enzyme's active site.

Given the values of ADP formation of more than 1000 min^{-1} in the first turnover for the VanA ligase, and if D-Ala$_1$-P is indeed an obligate intermediate in each catalytic cycle, then the PIX rate of 0.4 min^{-1}, although more than 40-fold over background, might be a dramatic underestimate. One possibility is that the presence of the second substrate D-Lac markedly accelerates catalysis; another is that the ADP in the ligase active site is in fact not free to rotate rapidly. The X-ray structure of ADP bound to the DdlB active site in complex with a phosphinophosphate transition-state analog indicates that there are contacts between the β-phosphate oxygens and backbone NH of Ser151, sidechains of Lys97 and Lys215, and two Mg^{2+} ions, which could hinder free rotation [15]. However, these residues are conserved in the StDdl D-Ala–Ala ligase, which had a much faster PIX rate of 600 min^{-1} [19]; thus, more structural information is needed to determine whether the β-phosphate of ADP is less able to rotate in VanC2 and VanA compared to StDdl. A third possibility is that ATP dissociation (e.g., of the observable β-^{16}O-γ bridged molecules) is slow, perhaps suppressed at high D-Ala$_1$ concentrations, and this might limit PIX rates. It may well be that PIX studies with [γ-^{18}O]-ATP, despite the advantage of not requiring release of ADP for detection of cleavage, will nonetheless underestimate the rates of formation of intermediates such as acyl phosphates by some members of this enzyme superfamily.

Additional methods could be used to further characterize D-Ala$_1$-P formation and build-up during the catalytic cycle of the D-Ala–D-X ligases. To date, all efforts to trap the D-Ala$_1$-P by quench into hydroxylamine have failed to detect the telltale D-alanyl hydroxamate (data not shown). The chemical synthesis of D-Ala$_1$-P and its evaluation for chemical and kinetic competence has yet to be reported. When we tried the more stable synthetic methyl ester of D-Ala$_1$-P, D-alanyl methyl phosphate (D-Ala$_1$Me-P) [38,39], it was not a substrate and only as a very weak inhibitor (K_I = 12 mM, data not shown), so the active site does not tolerate variation on the PO_3 moiety. Subsequent investigation should assess how the effective concentration and orientation of D-Ala$_1$-P and the D-Ala$_2$, D-Lac, and D-Ser in the D-Ala–D-X ligase active sites controls the unique product fluxes.

Significance

The D-Ala–D-X ligases, VanA and VanC2, are variants of the housekeeping bacterial enzyme D-Ala-D-Ala ligase that normally provides the D-,D-dipeptide for peptidoglycan termini. The altered specificity of VanA to make D-Ala–D-Lac and VanC2 to make D-Ala-D-Ser provide the new D-,D- metabolites that permit reprogramming of the peptidoglycan termini from D-Ala–D-Ala to D-Ala–D-Lac and D-Ala–D-Ser in the VanA and VanC phenotypes of vancomycin-resistant enterococci (VRE). To understand the mechanism and aid in the design of specific inhibitors of the D-Ala–D-X ligases, we have undertaken to validate that D-alanyl phosphate is a common intermediate that serves as the electrophilic substrate in the condensation with D-Ala$_2$, D-Lac or D-Ser as nucleophilic cosubstrates. The use of molecular isotope exchange (MIX), positional isotope exchange (PIX) and rapid quench kinetic studies are consistent with D-Ala$_1$-P intermediacy from both forward and back directions.

Materials and methods

Chemicals

NADH, NADP, L-lactate dehydrogenase (LDH), phosphoenolpyruvate and pyruvate kinase (PK) were from Roche Molecular Biochemicals. [^{14}C]-D-Ala, [^{14}C]-D-Lac, and [^{14}C]-D-Ser were from American Radiolabeled Chemicals Inc. [α-^{32}P]-ATP, [γ-^{32}P]-ATP were from New England Nuclear, and TLC cellulose and TLC polyethyleneimine cellulose plates were from Kodak. D-Ala–D-Ser was supplied by Advanced ChemTech. D-Ala, D-Ser, D-Lac, D-Ala–D-Ala, ATP, ADP, DL-alanine hydroxamate, hydroxylamine, glucose, hexokinase, glucose-6-P dehydrogenase, calf alkaline phosphatase, KH_2PO_4, buffers and other salts were purchased from Sigma. P-30 spin columns were obtained from Biorad. The [γ-$^{18}O_4$] ATP was prepared using the method of Moffatt and Khorana [40] from the respective morpholidate nucleotide precursor and $H_3P^{18}O_4$. The [γ-$^{18}O_4$]-ATP showed greater than 97% $^{18}O_4$ by ^{31}P NMR. D-Ala$_1$Me-P was synthesized as described by Kluger *et al.* [38] and shown to be ≥ 98 % pure by ^{1}H NMR. The phosphinate analog of D-Ala-D-Ala was a generous gift from Abbott Laboratories.

Protein purification

His-tagged VanC2 [14], His-tagged VanA [23] and untagged DdlB [27] were overexpressed and purified as described previously. The concentration of purified proteins was determined based on their absorbance at 280 nm and their extinction coefficients [41,42]. This method determined protein concentrations to be 2.8-fold higher for VanC2 and 2.2-fold higher for DdlB than determined previously using the Bradford method [12–14,27].

MIX

The MIX reactions included 100 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, D-Ala–D-Ser, 0.6 mM [^{14}C]-D-Ser (55 mCi/mmol), KH_2PO_4, 0 or 18 mM ADP, and 10 μM His-tagged VanC2. Control reactions excluded KH_2PO_4, D-Ala–D-Ser, or enzyme from the mixture above. The mixtures were analyzed on TLC cellulose plates to separate [^{14}C]-D-Ser and [^{14}C]-D-Ala–D-Ser [2]. The amount of D-Ala–D-Ser formed was quantified using a phosphoimager (BAS-1000 Fujix) and

corrected for nonenzymatic activity. The K_m for Pi was determined using 0.6 mM D-Ser and 150 mM D-Ala–D-Ser.

Reverse reaction by coupled assay

Steady-state kinetic constants for the reverse reaction of VanC2 were determined using a spectrophotometric assay monitored at 340 nm where the production of ATP is coupled to the reduction of $NADP^+$ through hexokinase and glucose-6-phosphate [19]. The reaction mixtures contained 20 mM MOPS, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 0.2 mM NADP, 2.0 mM glucose, 8 units of hexokinase, 4 units of glucose-6-P dehydrogenase, ADP, KH_2PO_4 and 1 μM VanC2 in a final volume of 1.0 ml. Negligible ATP was formed in the absence of ADP, KH_2PO_4, dipeptide or enzyme. The ADP K_m was determined in the presence of 50 mM D-Ala-D-Ser and 20 mM KH_2PO_4. For the measurement of dipeptide K_m values, 20 mM KH_2PO_4 and 2 mM ADP were used. For the KH_2PO_4 K_m value, 20 mM ADP and 67 mM D-Ala-D-Ser were included in the reaction mixture.

Forward reaction by coupled assay

Steady-state kinetic constants were determined using a spectrophotometric assay in which the production of ADP is coupled to the oxidation of NADH through PK and LDH [24,43]. For determination of the D-Ala_1 K_{m1} value for VanA at pH 6.0, VanA was incubated at 25°C with 200 mM MES, pH 6.0, 20 mM D-Lac, 5 mM ATP, increasing concentrations of D-Ala and the coupling reagents (2.5 mM phosphoenolpyruvate, 0.2 mM NADH and 50 units/ml of LDH and PK). The ATP K_m value for DdlB was determined using 100 mM HEPES, pH 7.5, 30 mM D-Ala, ATP and the coupling reagents. For comparison to the rapid quench results, the steady-state k_{cat} values for dipeptide/depsipeptide formation by DdlB and VanA were determined using the same buffer and substrate concentrations used for the rapid quench experiments plus the coupling reagents and 0.04 μM DdlB or 0.4 μM VanA.

PIX

For the PIX reactions, D-Ala, [γ-$^{18}O_4$] ATP, enzyme and buffer were incubated together at 30°C for 8 h. As controls, either enzyme or D-Ala was omitted. In addition, the full PIX reactions were repeated in the presence of either D-Lac or D-Ser and incubated until about 50% of the ATP remained (4.5 h). For the VanA reactions, all reactions mixtures contained 2 mM [γ-$^{18}O_4$] ATP, 100 mM MES, pH 6.0, 10 mM $MgCl_2$ and 10 mM KCl in a total volume of 0.75 ml. The amino/hydroxy acid and His-tagged VanA concentrations used are listed in Table 2. For the VanC2 reactions, the mixtures were the same as above except that HEPES buffer, pH 7.5, was used instead of MES buffer pH 6.0.

The full forward reaction was monitored by following the appearance of ADP spectrophotometrically in the lactate dehydrogenase/pyruvate kinase coupled assay [43], mentioned above. All reactions were quenched by addition of EDTA to a final concentration of 100–200 mM and analyzed by measuring the extent of exchange of the [β, γ]-bridge oxygen-18 within [γ-$^{18}O_4$] ATP with the β-nonbridge oxygen-16 by ^{31}P NMR on a Varian Unity 500 spectrometer with a 5 mm broadband probe operating at a frequency of 202 MHz. Typical acquisition parameters were: 10,000 Hz sweep width, 2.5 s acquisition time, 5 s delay, and a 30° pulse width.

Rapid quench

Rapid-quench reactions were performed at 30°C for various time points using a chemical quench flow instrument built by KinTek Instruments (Austin, Texas). For each time point, enzyme in 100 mM buffer (pH as listed in figure legends), 10 mM $MgCl_2$ and 10 mM KCl (buffer A) was loaded into one reaction loop (15 μl) and [α-^{32}P]-ATP (4 mCi/mmol) plus amino/hydroxy acids in buffer A were loaded into the second loop (15 μl). A computer-controlled motor drove syringes which forced the two reactants together and through the delay line. In the delay line, the reaction mixtures were incubated for the designated time (from 3 ms to 9 s) and then quenched with 500 mM EDTA (final EDTA concentration was about 330 mM compared with 3 mM $MgCl_2$) and expelled from the instrument giving a final volume of 100 μl. Alternatively, the reactions were quenched with 2N HCl, either 100 μl chloroform or 10 μl 0.5 M EDTA was added after expulsion, the solution was vortexed, and 20–22 μl of 2M Tris/3M NaOH was added to neutralize the reaction mixture [44]. If chloroform or EDTA was not added before neutralization, the enzyme was able to regain some activity upon neutralization and anomalously high activity was observed.

For DdlB, the results from the acid quench agree well with the results from the EDTA quench, implying that the burst of ADP is not an artifact due to slow chelating of enzyme bound Mg^{2+} by EDTA allowing formation of ADP after quenching. As a control for contaminating ATPase activity, the reactions were performed in the absence of amino/hydroxy acid substrates. Also, a negligible amount of ADP was formed when enzyme was reacted with substrates plus EDTA (data not shown). The reaction mixtures were analyzed on TLC cellulose-polyethyleneimine plates to separate [α-^{32}P]-ATP and [α-^{32}P]-ADP using 0.75 M potassium phosphate, pH 3.5, for development [26] and quantitated using a phosphoimager.

Determination of active DdlB and VanA concentrations

In triplicate, 38 μM DdlB was incubated with 1 mM [$\gamma^{32}P$]-ATP (10 mCi/mmol) and 310 μM D-Ala-D-Ala phosphinate inhibitor (K_I = 33 nM [27]) in 100 mM HEPES, pH 7.5, 10 mM $MgCl_2$ and 10 mM KCl (buffer B) for 1 h at room temperature to generate enzyme bound [^{32}P]-phosphorylated inhibitor. The enzyme–inhibitor complex was separated from free [$\gamma^{32}P$]-ATP by using a P-30 spin column. The amount of DdlB active for phosphoryl transfer was determined by measuring the per cent of the total counts, and thus the percent of ATP, that eluted from the spin column minus the 0.06% of ATP that eluted in the absence of enzyme or inhibitor. As additional controls, more than 95% of the enzyme was eluted from the column and only 1.3% of the starting enzyme activity remained after incubation with the inhibitor and ATP. This residual activity was measured by incubating enzyme and unlabeled ATP with or without inhibitor for 1 h, passing the mixtures through P-30 spin columns to remove free ATP and inhibitor, and then comparing the amount of ADP produced by each mixture in the presence of 50 mM D-Ala and 5 mM ATP in buffer B using the lactate dehydrogenase/pyruvate kinase coupled assay [43].

For VanA, the above protocol was used with 500 μM phosphinate inhibitor (K_I = 4.1 μM [45]). As controls, only 0.1% of the ATP eluted from the column in the absence of inhibitor or VanA enzyme, and only 0.7% of the enzyme activity remained after incubation with the inhibitor and ATP.

D-Ala_1Me-P inhibition

Inhibition of D-Ala-D-Lac synthesis by 0–100 mM D-Ala_1Me-P was tested for 0.6, 2, 5 and 10 mM D-Ala using 13 μM VanA, 20 mM D-Lac and 5 mM ATP in 200 mM MES, pH 6.0, 10 mM $MgCl_2$, and 10 mM KCl. ADP production was measured in the lactate dehydrogenase/pyruvate kinase coupled assay [43]. K_I and IC_{50} values were determined using a K_{m1} value of 3.24 mM and equations 12 and 13 for pure competitive inhibition against Ala_1 [46]:

$$1 - \frac{v_i}{v_o} = \frac{[I]}{[I] + K_I\left(1 + [S]/K_m\right)} \qquad (12)$$

$$IC_{50} = \left(1 + \frac{[S]}{K_m}\right)K_I \qquad (13)$$

where v_i and v_0 are the velocities in the presence and absence of inhibitor, respectively.

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