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Role of the omega loop in specificity determination in subsite 2 of the D-alanine:D-alanine (D-lactate) ligase from *Leuconostoc mesenteroides*: A molecular docking study

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ABSTRACT

The synthesis of D-ala-D-lactate in *Leuconostoc mesenteroides* is catalyzed by D-alanine:D-alanine (D-lactate) ligase (ADP). The ability to assemble this depsipeptide as well as D-ala-D-ala provides a mechanism for the organism's intrinsic resistance to vancomycin. Mutation of Phe261 to Tyr261 in the Ω-loop of this ligase showed a complete loss of the ability to make D-ala-D-lactate (Park and Walsh, J. Biol. Chem. 272 (1997) 9210–9214). Phe261 is a key specificity determinant in the α-helical cap of the Ω-loop when folded into the closed conformation. A molecular docking study of the closed ligase using AutoDock 4.2 defines additional specificity constraints promoted by the Ω-loop capping the catalytic center.

Attaining productive orientations of D-lactate with favorable ligation chemistry requires the flexibilities of Phe261 and Arg301 in the docking protocol. These are in addition to the optimization of van der Waals contacts with Lys260, Met326, and Ser327. The location of Phe261 and Lys260 in the α-helical cap of the Ω-loop over subsite 2 is an essential part of the folding process ensuring depsipeptide formation in the hydrophobic environment of the catalytic center. The importance of the F261Y mutation suggests that the hydroxyl of Tyr261 plays an instrumental role in determining non-productive docking orientations of D-lactate. Two of these are presented: (A) D-lactate-OH as an H-bond donor to the Tyr261-OH; (B) D-lactate as an H-bond donor to the phosphoryl of the intermediate D-alanyl phosphate, and the D-lactate-COO^- as an H-bond acceptor for the Tyr261-OH. Neither orientation, A or B, show the bifurcated H-bonding with Arg301 recently proposed for the activation of the nucleophilic D-lactate for D-ala-D-lactate formation. Insights into the role of the Ω-loop and its K(F/Y) signature provide additional background for inhibitor design targeted to subsite 2 of the D-alanine:D-alanine (D-X) ligases.

1. Introduction

D-Alanine:D-alanine (D-lactate) ligase (ADP) from *Leuconostoc mesenteroides* catalyzes the synthesis of the depsipeptide, D-alanyl-D-lactate, in addition to D-alanyl-D-alanine, when D-alanine and D-lactate are incubated together. The depsipeptide is responsible for the intrinsic resistance of this organism to vancomycin. This glycopeptide antibiotic binds with a greatly reduced affinity (1000-fold) to cell wall intermediates that terminate in acyl-D-ala-D-lactate when compared to acyl-D-ala-D-ala [1–3].

The focus of the docking studies reported in this paper is subsite 2 in the D-alanine:D-alanine (D-lactate) ligase from *L. mesenteroides* and those amino acid residues which determine the specificity of the second stage in the ligation reaction. Evidence for this subsite is based on kinetic studies [4], mutagenesis experiments [5–7] sequence alignments [8–11] inhibitor studies [12–15], mechanism studies [16] and crystallographic measurements [10–12,17–20]. The molecular analysis of the ligase showed both closed (monomer 1) and open (monomer 2) conformers in the unit cell. The closed monomer contains a phosphoryl-phosphinate analog of the reaction intermediate in the presence of Mg^{2+} and ADP. The open monomer is devoid of these ligands [18].

In the formation of the closed structure, the three (α + β) domains and Ω-loop fold to form a hydrophobic cavity that surrounds the ATP-grasp domain and the catalytic center [17–20,22]. The transition resulting in this closed cavity occurs when inhibitor **1** [1(*S*)-amino-ethyl (2-carboxy-2(*R*)-methyl-1-ethyl)-phosphinic acid] is phosphorylated by ATP in the presence of Mg^{2+} and results in the ordering of the Ω-loop over the cavity. Folding places a short two-turn α-helical cap or lid over the catalytic center with the R groups of Phe261 and Lys260 directed to subsite 2 [18], the focus of this paper.

The phylogenetic tree of D-alanine:D-alanine ligases includes at least 5 groups [6,8,9]. These are: (1) D-alanine:D-alanine (D-lactate)

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ligases (lactic acid bacteria), (2) D-alanine:D-alanine ligases (DdlA group from *Escherichia coli*), (3) D-alanine:D-alanine ligases (DdlB group from *E. coli*), (4) D-alanine:D-alanine (D-lactate) ligases (vancomycin inducible, VanA and VanB), and (5) D-alanine–D-serine ligases (VanC group). All have the similar or conserved Ω-loop motif –(255)YXYXXK(F/Y)– found in *L. mesenteroides* with the exception of group 4 [7–9]. For purposes of this paper the K(F/Y) consensus is termed the signature of this motif. VanC has only this conserved KY [8]. In addition, the thermophile subfamily (*Thermus caldophilus* and *Thermus thermophilus*) also contains the conserved motif in the α-helical segment [19,20]. The exceptions, VanA and VanB [21], have a different motif and thus, do not contain the KF/Y signature. The Ω-loop of VanA differs in both structure and composition from those ligases with the KF/Y signature [21]. Exchanging the Ω-loop of the VanA ligase with that of the VanC ligase inactivated D-ala-D-lactate formation and increased by 3-fold the synthesis of D-ala-D-ala and D-ala-D-ser [6]. In the ligases that only synthesize D-ala-D-ala, e.g. the D-alanine:D-alanine ligase B from *E. coli*, the cap of the Ω-loop has Tyr216 in place of the homologous Phe261 in the *L. mesenteroides* D-alanine:D-alanine (D-lactate) ligase. In addition to these residues, Tyr255 and Tyr257 are also conserved in the alignments. Thus, the consensus sequence of the α-helical cap of the Ω-loop is –(255)YXYXXK(F/Y)– [8,11]. With the exception of Tyr255, all residues of the motif are in the α-helical cap in those ligases which have been examined (*L. mesenteroides* [18], *E. coli* DdlB [17], *Helicobacter pylori* [11], *T. thermophilus* [19]).

Mutagenesis of Phe261 in the *L. mesenteroides* ligase to tyrosine results in the complete loss of depsipeptide activity while enhancing the D-alanine:D-alanine ligase activity (k_{cat}/K_m) by 3-fold [7]. Based on this mutagenesis result it was proposed that Phe261 is "a molecular indicator of both the ability to make D-ala-D-lactate and its intrinsic resistance to vancomycin" [7]. Mutagenesis of Tyr216 to phenylalanine in the *E. coli* ligase B enhances the D-alanine:D-lactate ligase activity [23]. Thus, ordering of the Ω-loop in the closed monomer of the *L. mesenteroides* ligase with its phenylalanine residue in the cap is an important folding event covering the reactants as well as orienting this residue that is instrumental in defining the ligation of D-alanine and D-lactate.

Previous docking studies [24] with the D-alanine:D-alanine (D-lactate) ligase from *L. mesenteroides* without the Ω-loop (residues 247–267) and serine–serine loop (residues 185–188) suggested that Arg301 has a dual function in a sequential reaction mechanism, i.e. substrate orientation and nucleophile activation in subsite 2 as well as stabilization of the transition state complex. In flex-flex docking, the guanidino group of this Arg residue rotates to form a bifurcated H-bond with the D-lactate-OH. Although this docking study provided an insight into the mechanism of nucleophile activation, the absence of the Ω-loop did not allow a definition of the roles for Phe261 and Lys260 in the motif –YXYXXKF–.

It is the goal of my studies to recapitulate the specificity determinants of the complete *L. mesenteroides* ligase that determine the docking poses of D-alanine and D-lactate in subsite 2. In order to define which residues form van der Waals contacts, interaction displays with key residues in this site will be examined with particular emphasis on Phe261 and the Tyr residue in the F261Y mutant. Correlation between the present studies and those described previously [24] provides a basis for defining those residues in subsite 2 that are instrumental in determining the specificity in the second stage of the ligation reaction.

2. Methodology

2.1. Software and PDP files

AutoDock 4.2 and AutoDockTools 4 (ADT) were chosen for these studies [25,26]. A crystal structure of D-alanine:D-alanine (D-lactate) ligase (ADP) from *L. mesenteroides* (PDB ID: 1EHI) [18] was retrieved from the RCSB Protein Data Bank. Other software used in this study was CAVER [27,28], DynDom3D [29,30] and Swiss PdbViewer [31].

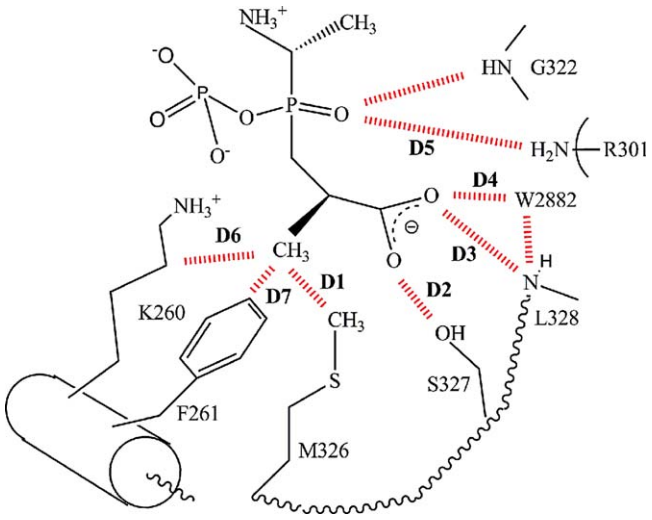

Fig. 1. Schematic identifying the distances of the transition-state analog, phosphoryl-phosphinate **2** to key residues of the ligase listed in Table 1. D6 is the distance between the δ-methylene of Lys260 and the D-lactate-CH_3. D7 is the distance between the 4-carbon of Phe261 and D-lactate-CH_3. D2 represents the distance between the oxygen atoms of Ser327 and the $-COO^-$ of phosphoryl-phosphinate **2**.

The PDB file for the D-alanine:D-alanine (D-lactate) ligase contains the coordinates for monomers A (closed) and B (open) of the unit cell (Protein Data Bank) and they are referred to as monomers 1 and 2, respectively, by Kuzin et al. [18]. Co-crystallization of the protein with ATP and 1(*S*)-amino-ethyl (2-carboxy-2(*R*)-methyl-1-ethyl)-phosphinic acid (**1**) resulted with the transfer of the γ-phosphoryl of ATP to the phosphinic acid **1** in monomer A yielding the transition-state analog, phosphorylated-phosphinate **2** (Fig. 1).

Several modifications were made to the PDB file of monomer A. First it was observed that a water bridges between the carboxylate of phosphorylated-phosphinate **2** (Fig. 1) and the backbone amide of Leu328 [18]. This water was renamed W2882 and retained for all docking experiments. All other water molecules were removed. Second, after energy minimizing in Swiss-PdbViewer, the phosphoryl-phosphinate **2** was converted to enzyme-bound D-alanyl-phosphate in subsite 1. Other modifications are described in Ref. [24]. Using monomer A has the advantage of preserving the catalytic and specificity architecture of the amino acid residues that determine subsites 1 and 2. The *L. mesenteroides* mutant ligase F261Y was constructed in Swiss-Pdb Viewer.

2.2. Flex-flex docking

The choice of AutoDock 4.2 was based on the ability to program the flexibility of specific residues in the receptor protein as well as the ligand. The bonds in D-lactate (C^α–OH and C^α–COO^-) were allowed to rotate and the flexibility built into the PDBQT files with the use of ADT. The previous study [24] indicated that flexibility of the guanidino group of Arg301 was essential for assisting nucleophile activation and orientation of D-lactate. In these docking experiments, variable torsion (bond between C^δ and N^ε) of Arg301was programmed in all docking protocols. For other protocols that include flexibility in Phe261, Lys260, and Tyr261 (mutant ligase F261Y) the flexible bonds are detailed in the figure and table legends. If all residues of the receptor protein were held rigid, the docking was termed flex-rigid docking. Distance measurements in

subsite 2 docking poses were made with ADT and schematically indicated in Fig. 1.

2.3. Docking protocol

The basic docking protocol was adapted from Neuhaus [24] using the default settings provided by ADT [26]. The protein structure (PDB format) was converted to PDBQT format in ADT. Since AutoDock 4.2 uses a grid-based algorithm and not a trajectory-based program to evaluate trial conformations, docking can occur on either surface receptor sites or in internal cavity sites of the protein. The grid size for the search space was set at $46 \times 46 \times 46$ centered in the subsite 2 cavity with a default grid point spacing of 0.375 Å. In contrast, the previous studies [24] used a grid size of $20 \times 20 \times 20$. The enlarged size ensured that the docking cavity included the Ω-loop and the additional cavity space detected in the CAVER analysis (Fig. 3). The Lamarckian genetic algorithm was used with a population size of 150 dockings and 5 million energy evaluations. For visualizing atom–atom contacts in docking poses with the ligand and residues of subsite 2 in MGL tools, a VDW scaling factor of 1.0 was used to ensure that only those atoms in contact with the ligand are shown.

3. Results

3.1. Role of the omega loop

The α-helical segment of the Ω-loop (residues 257–262) in monomer A is shown covering subsite 2 of the catalytic center (Fig. 2). When the phosphoryl-phosphinate **2** (Fig. 1) is formed, the open form of the ligase (monomer B) undergoes a series of folding events that result in the α-helical cap and ordering of the Ω-loop [18]. The ordering of this loop with the cap positions Phe261 and Lys260 onto subsite 2. Monomer B lacking ADP, $Mg^{2+,}$ and the phosphorylated-phosphinate **2** does not undergo this folding process. The rotation angle between the fixed and moving domains in these monomers is 8.9° and the closure is 88% in monomer A [29,30]. Closure is based on the relationship between the hinge axis and the line joining the centers of mass of the two domains [32]. This movement is accompanied by the formation and targeting of the α-helical cap to subsite 2 of the catalytic center.

The ligase docking cavity (subsite 2) is formed by the removal of the 2-carboxy-2-methyl-1-ethyl moiety of **2** in monomer A. The resulting cavity is visualized at the confluence of two tunnels (Fig. 3A and B). For comparison, Fig. 3A shows the subsite 2 cavity

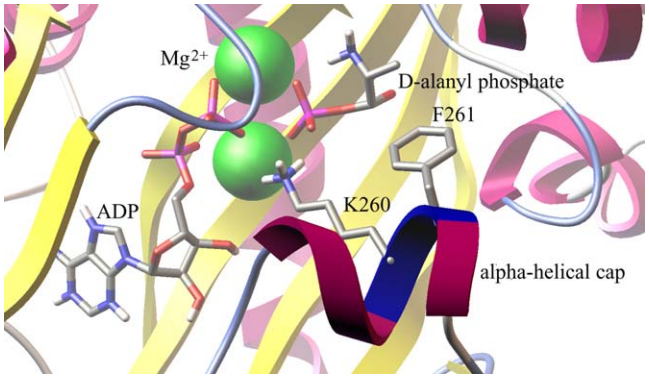

Fig. 2. α-Helical cap over the docking cavity of the D-alanine:D-alanine (D-lactate) ligase with Phe261 and K260 identified. The α-helical segment is composed of (257)YNNKFV.

with Lys260 and Phe261 bounding the tunnel. In Fig. 3B Met326 and Ser327 are visualized to illustrate their definition of the cavity. The docking experiments described in the present studies address the cavity encompassing only subsite 2 and capped by the α-helical fragment described above.

3.2. Docking poses of D-lactate

To identify optimal D-lactate docking poses with the ligase, favorable features of the reaction chemistry and distance measurements from the crystallographic structure were used as criteria for productive poses. These included: (1) a nucleophile–electrophile distance of 3.2 ± 0.2 Å, (2) a mechanism of nucleophile activation, (3) a mechanism of D-lactate-OH deprotonation, and (4) optimization of the van der Waals contacts with Lys260, Phe261, Met326, and Ser327. Attaining productive poses requires the flexibilities of the key residues, Arg301 and Phe261. Poses that do not satisfy these criteria were determined to be non-productive. The use of AutoDock 4.2 with both ligand and selected receptor residue flexibilities is ideally suited for studying this problem.

In Fig. 4, a pose of D-lactate that meets these criteria is shown. The guanidino group of Arg301 rotated 37° to accommodate the bifurcated H-bonding to the D-lactate-OH. The bifurcated H-bonding and the deprotonation to the phosphoryl oxygen of D-alanyl phosphate provide a mechanism for activating the nucleophile. Based on the mutagenesis experiments of Park and Walsh [7], it was predicted that Phe261 will play a major role as a speci-

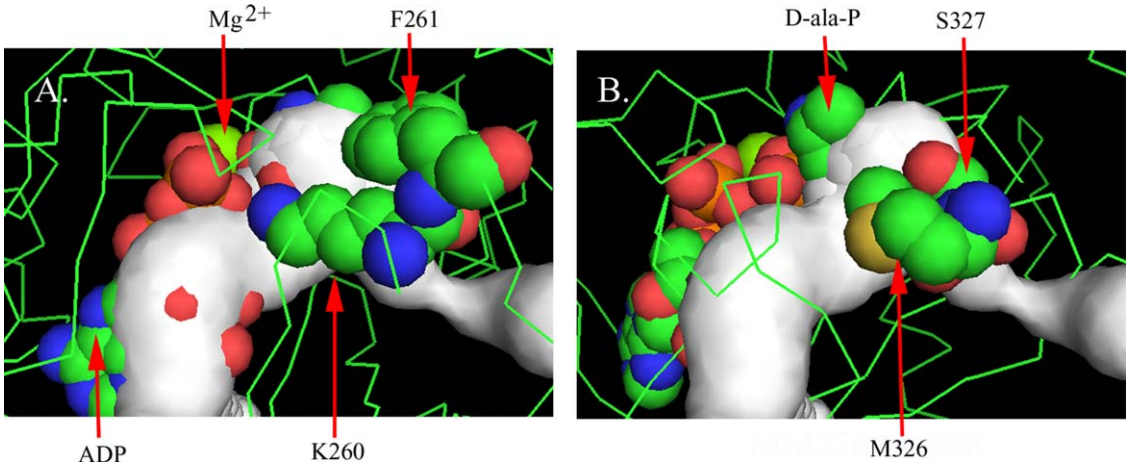

Fig. 3. Analysis of the docking cavity with Phe261 and Lys260 (A), and Met327 and Ser326 (B). Five tunnels were computed with D-alanyl-phosphate as the starting point with CAVER [27,28] and the two that are bounded by these residues and ADP are shown. B is rotated −45° to visualize the interactions of the Met327 and Ser326.

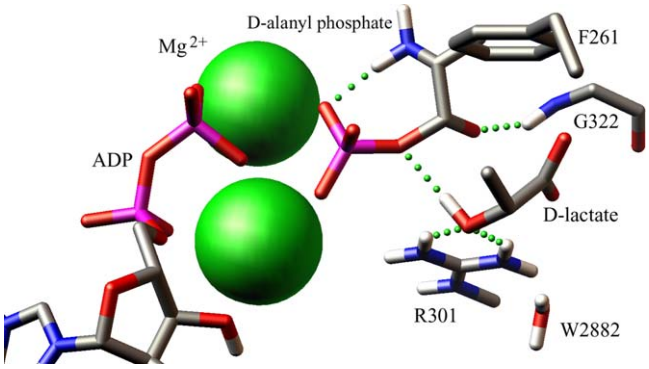

Fig. 4. Productive orientation of D-lactate in flex-flex docking. For this docking protocol, the complete ligase was used with F261 and R301 as flexible residues. H-bonding with Gly322 defines the oxyanion hole. The choice of this pose is based on the favorable chemistry described in the text. For F261, the variable torsions are C^{α}–C^{β} and C^{β}–C^{1}.

ficity determinant and, thus, this residue was also designated as flexible. Although Lys260 had not been identified as a specificity determinant, it was observed to form van der Waals contact with D-lactate in this docking pose. Thus, an additional specificity role of the K(F/Y) signature was considered.

3.3. Atom interactions of Phe261 and Lys260 with D-lactate

The interaction displays of van der Waals contacts are shown in Fig. 5A and B in two perspectives. These interactions correlate with those established for the phosphoryl-phosphinate **2** as well as those established for D-lactate in the ligase minus the Ω-loop (Table 1). As shown in Fig. 5A, C-4 of the phenyl- in Phe261 contacts the CH_3- of the D-lactate when Phe261 and Arg301 are flexible. Contact was not observed if Phe261 is rigid. A second interaction occurs with the δ-methylene of Lys260. To optimize the interactions, a docking protocol with flexible Lys260, as well as Arg301 and Phe261, was performed As shown in Table 1 (column B), only modest improvements in the distance measurements (D1 and D3) were observed. These interactions with residues of the Ω-loop, together with the van der Waals contacts of D-alanyl phosphate, Met326, Ser327, R301 and W2882, define the docking cavity for D-lactate.

Table 1
Distances (Å) in subsite 2 for docking poses of D-lactate.

Distance (Å)[a]	Phosphoryl-phosphinate **2**-ligase[b]	D-Lactate[c]	A F261	B F261 K260
D1	3.5	3.7	3.7	3.5
D2	2.6	2.9	2.9	2.8
D3	4.0	4.0	4.5	3.9
D4	2.8	2.9	3.3	3.0
D5	3.0	3.8	3.8	3.8
D6	4.0	–[d]	3.6	3.6
D7	4.3	–[d]	3.6	3.6
O→C	–[d]	3.1	3.2	3.0

[a] Distances for D-lactate docking (A) were measured on the pose (Fig. 4) in flex-flex docking with Arg301 and Phe261 as flexible residues. The distances in (B) reflect a docking pose (not shown) where the flex residues included Lys260. See Fig. 1 for the schematic identifying these distances. The variable torsions for Lys260 under (B) are C^{α}–C^{β}, C^{β}–C^{γ}, C^{γ}–C^{δ}, and C^{δ}–C^{ε}.
[b] D1–D7 were calculated from the coordinates of the co-crystallized phosphoryl phosphinate **2**-ligase [18].
[c] Docking to ligase without Ω-loop and Ser-ser loop [24].
[d] Not relevant.

3.4. Docking poses of D-alanine in cavity

No docking poses that satisfy the criteria presented above were observed with the entire ligase and D-alanine. However, since the cavity is determined by the removal of the 2-carboxy-2-methyl-1-ethyl moiety, it is reasonable to propose that docking of D-alanine may be restricted by this cavity. To observe productive D-alanine docking poses, the signature residues, Phe261 and Lys260, had to be deleted before a docking pose that meets the criteria described above was observed (Fig. 6). In this pose the guanidino group of Arg301 rotated to form one H-bond with the D-alanine-COO^{-}. This orientation allowed the protonated $-NH_3^{+}$ of D-alanine to form two H-bonds with the phosphoryl of D-alanyl-phosphate. The N→C distance is 3.5 Å, and these H-bonds allow for nucleophile activation and deprotonation. It is noted that Tyr255, a residue in the –(255)YXYXXKY– motif, also plays a role in defining D-alanine docking (Fig. 6B).

3.5. The F261Y mutation

The importance of the F261Y mutation as a "molecular indicator of D-ala-D-lactate formation" [7] suggests that the –OH of Tyr261 plays an instrumental role in determining a non-productive orientation of D-lactate. The fact that only mutation of Phe261 to Tyr261

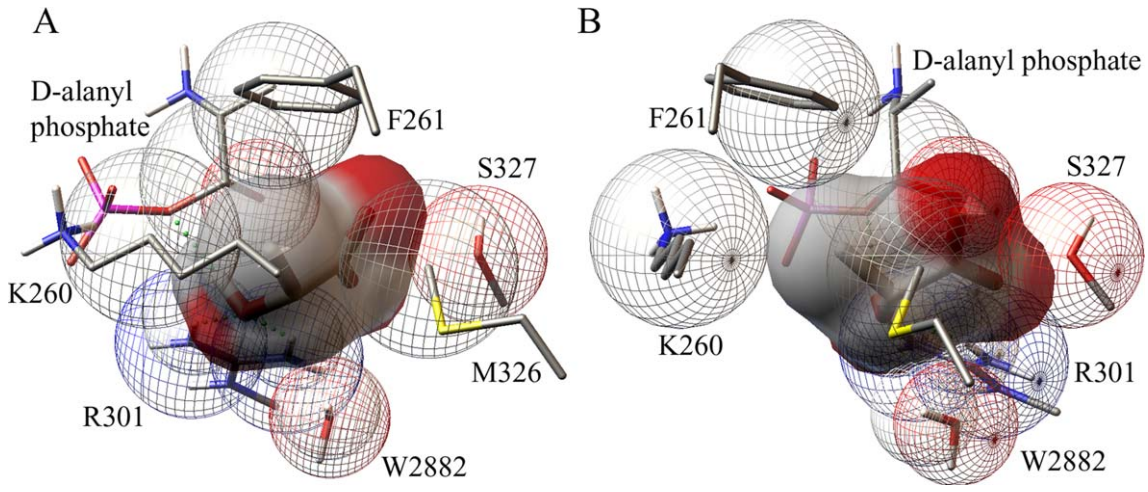

Fig. 5. Interaction displays of D-lactate in two perspectives. Perspective A is that from Fig. 4. B is rotated +45° relative to A to illustrate the contacts of Phe261 and Lys260 with the D-lactate. D-Lactate is shown in msms (maximal speed molecular surface) format.

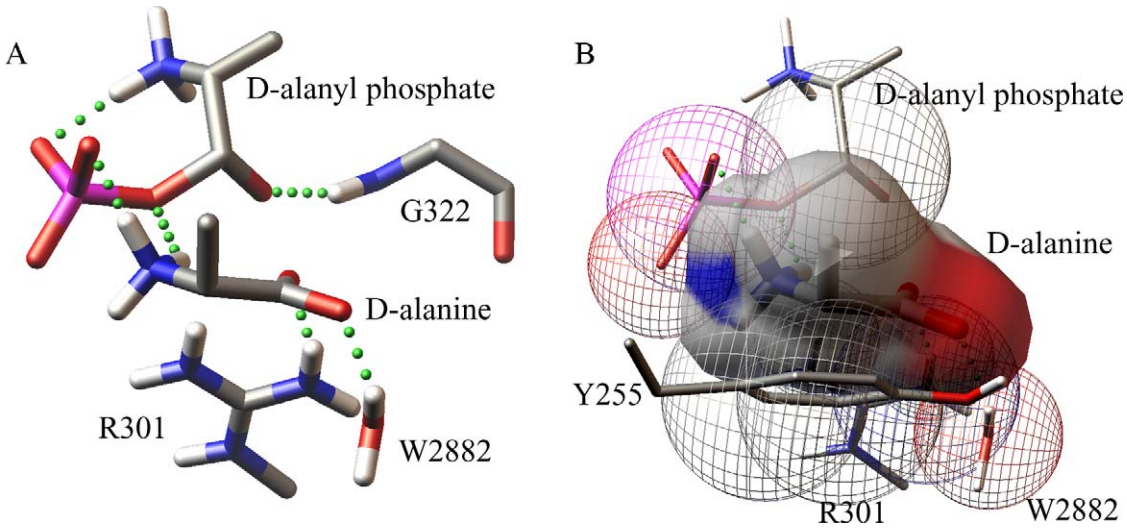

Fig. 6. Orientation of D-alanine (A) and interaction display (B) in the subsite 2 cavity. Phe261 and Lys260 were deleted from the ligase and Arg301 is flexible. In B, D-alanine is presented in msms format.

conveys this marked change in specificity strongly suggests that the tyrosine –OH has major effect. In examining the mutant ligase (F261Y), flexibility was programmed in both Tyr261 and Arg301. While the orientations shown in Fig. 7A and B are in addition to other productive and non-productive poses, they are the only ones characteristic of the mutant ligase. Each of these poses is characterized by the H-bonding of the tyrosine –OH residue, both as a donor and as an acceptor (Fig. 7A and B), and are not observed when the ligase with the KF signature is used (see above). Thus, since these poses are unique to the mutant ligase, it was proposed that they represent manifestations of the mutant ligase that allow us to recapitulate, in part, the mutagenesis experiment described by Park and Walsh [7].

The analysis of residue interactions in orientation A (Fig. 8A) shows van der Waals contacts with Tyr255, Lys260, Tyr261, and Met326 of the mutant ligase. For orientation B (Fig. 8B) the interactions are with Tyr255, Lys260 and Tyr261. This interaction with Tyr255 was not observed with the favorable orientation found for D-lactate (Fig. 4). Although these orientations in Fig. 7 would not be productive, there is an additional feature of orientation A that may play a role in determining the switch from D-ala-D-lactate to D-ala-D-ala formation. The change from Phe261 to Tyr261 provides a donor proton for H-bonding to Ser186. This H-bonding is homologous to that in the *E. coli* ligase between Tyr216 and Ser150 of the Ser-Ser loop [17]. Because of the importance of this tyrosine in the *E coli* ligase B and its counterpart in the *L. mesenteroides* F261Y mutant, it is possible that this H-bonding may also play a role in the molecular switching proposed by Park and Walsh [7].

4. Discussion

The Ω-loop with its two-turn α-helix is an important architectural feature of the *L. mesenteroides* D-alanine:D-alanine (D-lactate) ligase in the closed conformation. The K(F/Y) signature in the motif of this loop plays an essential role in determining the specificity of the enzyme. In addition, this two-turn α-helix provides a cap over subsite 2 of the catalytic center protecting the reactants from hydrolysis. The α-helical cap with its KF signature is positioned in part by two H-bonds to the serine-serine loop (185–188) in the *L. mesenteroides* ligase, In the *E. coli* ligase B three H-bonds are possible with its KY signature [7].

In Table 1 the distance measurements for D-lactate and subsite 2 residues are summarized with atom-atom center distances. These are compared with the distances determined from the phosphorylphosphinate **2** transition-state analog and correlate well with the docking studies previously published [24] for the ligase minus the Ω-loop and the serine-serine loop. While there is not convergence among all the docking poses in these studies, the poses were cho-

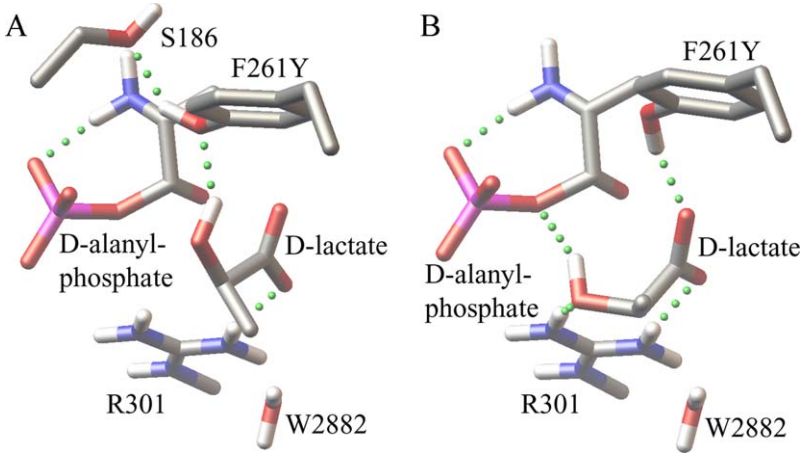

Fig. 7. Non-productive orientations, A and B, characteristic of the F261Y mutation. In A, Tyr261 is an H-bond acceptor from D-lactate and in B, it is an H-bond donor to the D-lactate-COO^-. In A, Tyr261 also H-bonds with Ser186. The variable torsions in Tyr261 are: C^{α}–C^{β}, C^{β}–C^{1}, and C^{4}–OH.

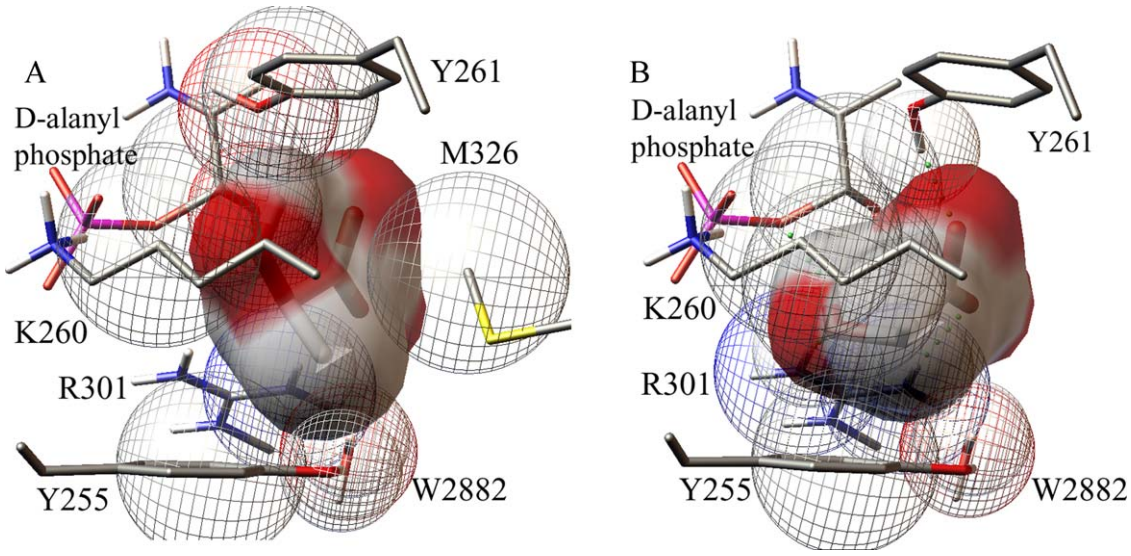

Fig. 8. Interaction displays of the non-productive D-lactate orientations, A and B (Fig. 7), in the F261Y mutant. D-Lactate is shown in msms format.

sen based on the chemistry: mechanism of nucleophile activation (bifurcated H-bonding with Arg301), nucleophile–electrophile distance, and ligand deprotonation. Each of these criteria was satisfied before the pose was chosen. Seven distances define the docking of D-lactate in subsite 2. They are with Lys260, Phe261, Arg301, Met326, Ser327, Leu328, W2882, and D-alanyl phosphate. In the crystallographic structure of monomer A, Ser327 forms a H-bond with the phosphoryl-phosphinate **2**-COO^- [18].

The D-alanine:D-alanine (D-lactate) ligase has a catalytic efficiency ratio of ~3/1 in favor of depsipeptide over D-ala-D-ala [7]. Thus, it was unexpected that no docking orientations of D-alanine were observed with the complete ligase. However, the docking orientation of D-alanine in the ligase minus the Ω-loop and ser-ser loop gave satisfactory poses consistent with the mechanism of deprotonation, the N→C distance of 3.2 Å and van der Waals contacts with Met326, Ser327 and Arg301 [24]. Only after deletion of both Phe261 and Lys260 of the complete ligase was a docking orientation observed that satisfied the chemistry of the ligation reaction (Fig. 6). The observation that D-alanine does not dock with a productive orientation in the complete ligase introduces a caveat with the docking experiments that utilize the complete protein. The folding and ordering of the Ω-loop is determined by the phosphoryl-phosphinate **2** formed from phosphinate **1** and ATP during crystallization [18] In the absence of the Ω-loop [24], removal of the 2-carboxy-2-methyl-1-ethyl moiety was sufficient because of the additional space provided by the removal of the Ω-loop. Thus, subtle folding changes in the Ω-loop to accommodate the phosphoryl-phosphinate inhibitor **2** may differ from that which would occur if D-alanine (or D-ala-D-ala), were co-crystallized with the protein.

The primary role of the ε-amino of Lys260 is to H-bond with the phosphoryl of the phosphoryl-phosphinate **2** and Ser187-OH [18]. During these docking studies, it was also observed that δ-methylene carbon of this lysine contacts with the D-lactate-CH_3. Thus, Lys260 in the α-helical cap plays an additional role as a specificity determinant for defining the docking site for D-lactate. The aliphatic chain of the homologous Lys in the *T. thermophilus* ligase also makes contact with the methyl group of the second D-alanine [19]. The third residue of the cap that plays a role is Tyr255 of the motif [18]. The H-bond between the amide carbonyl of this residue and Ser187 functions in the ordering and assembly of the cap over the cavity. Thus, these residues, together with Phe261 play roles in determining the specificity of the ligation reaction.

The attempt to recapitulate the molecular switch in the mutant (F261Y) ligase has been only partially achieved. The observed orientations that are correlated with the replacement of Phe261 by Tyr261 are non-productive and dependent on the tyrosine-OH. These H-bond arrays promote non-productive orientations that would appear to be in competition with the productive ones. However, D-lactate has not been reported to be a competitive inhibitor of D-ala-D-ala formation in the mutant ligase.

In limited proteolysis studies of the D-alanine:D-alanine ligase B from *E. coli*, it was discovered that ATP and phosphinate **1** conferred complete protection to the enzyme [15]. It was proposed that this protection resulted from freezing an insensitive enclosed enzyme conformation. This transition from an open to closed conformation is a type of induced fit mechanism to ensure efficient transfer of the γ-phosphate of ATP to D-alanine for formation of the D-alanyl phosphate intermediate in subsite 1 of the ligase [15,16]. Co-crystallization of the D-alanine:D-alanine ligase B with phosphinate **1** and ATP with the formation of phosphoryl-phosphinate **2** resulted in the ordering of the Ω-loop (206–220) with its α-helical cap over the catalytic cavity [17]. Solvent accessibility calculations showed that only 2% of the ADP and phosphoryl-phosphinate **2** in the cavity is exposed to the medium [12,17]. These studies provided the basis for understanding the role that the Ω-loop plays in shielding the intermediates from water.

An analysis of the transition from open to closed conformations in D-alanine:D-alanine ligase from *T. thermophilus* HB8 has provided additional insights [19]. In this study, a series of cumulative conformational changes were observed in four co-crystallizations: (1) no ligand, (2) ADP, (3) D-alanine and ADP, and (4) D-ala-D-ala and ATP. These conformations are designated: (1) open, (2) semi-open, (3) semi-closed, and (4) closed [19], and are characterized by the rotation angle of the central domain of the ligase. In the semi-closed conformation with ADP and D-alanine, the Ω-loop is ordered but the rotation is not complete. In an analysis of the D-alanine:D-alanine ligase from *T. caldophilus*, Lee et al. [20] characterized the closure as the rotation of the central domain (17°) and the swing motion of the lid (cap) to the closed conformation upon substrate binding. Thus, complete closure of the ligase is not achieved until both subsites 1 and 2 are occupied with substrate together with ATP and Mg^{2+}.

The conformational changes resulting from the binding of D-alanine, D-lactate, ATP, and Mg^{2+} are an excellent example of induced fit [15,33]. Not only does this process bring catalytic groups

into the proper alignment, but induced fit may also play an important role in determining ligase specificity. The rate at which the enzyme opens to release bound substrate is a kinetic property [34]. In the case of the ligase, slow dissociation of substrate from subsite 2 in the folded conformer would be consistent with a good substrate while fast dissociation would be consistent with a poor substrate [34]. This interpretation may be correlated with the proposal of Healy et al. [8] that the Tyr/Phe switch is an architectural element for dipeptide to depsipeptide formation.

D-Alanine:D-alanine (D-X) ligases are members of the ATP-grasp superfamily that include glutathione synthetase [35], biotin carboxylase [36] and carbamoyl phosphate synthetase [37]. Each of these has an Ω-loop [38] and has a similar mechanism of ATP utilization in the ligation reaction. This loop ensures the specificity of the acceptor substrate and determines solvent accessibility to the catalytic center.

The conclusions resulting from my study of the D-alanine:D-alanine (D-lactate) ligase from *L. mesenteroides* can be correlated with the structure studies of the *E. coli* D-alanine:D-alanine ligase B [12,17] and the two thermophile ligases [19,20]. Each of these has the short α-helical cap upon ordering of the Ω-loop with the YXYXXK(F/Y) motif. However, the results with the Ω-loop of these ligases are not easily extrapolated to either the inducible VanA or VanB ligases. While VanC (D-alanine:D-serine ligase) has the KY signature, the remainder of the motif shares no additional consensus with the motif described in this paper. To achieve a more complete understanding of this switching, a molecular dynamics approach may be necessary that focuses on the dynamics, folding, and ordering of the Ω-loop with D-alanine or D-ala-D-ala as ligands. Understanding the mechanism of these ligases and their role in determining resistance to vancomycin is important in our development of new strategies of antibiotic design that could overcome resistance to this antibiotic.

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