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Role of Arg301 in substrate orientation and catalysis in subsite 2 of D-alanine: D-alanine (D-lactate) ligase from *Leuconostoc mesenteroides*: A molecular docking study

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ABSTRACT

D-Alanine:D-alanine (D-lactate) ligase (ADP) from *Leuconostoc mesenteroides* synthesizes the depsipeptide, D-alanyl-D-lactate, in addition to D-alanyl-D-alanine, when D-alanine and D-lactate are incubated simultaneously. The depsipeptide is responsible for the intrinsic resistance of this organism to vancomycin. The orientations of D-lactate and D-alanine in subsite 2 of the ligase that result in both nucleophile generation and subsequent attack on the electrophilic center of D-alanyl phosphate in subsite 1 are not known. A molecular docking study using AutoDock 4 suggests a role for Arg301 in determining these orientations of acceptor substrate in subsite 2 for both nucleophile generation and subsequent attack on the phosphate intermediate.

With D-lactate a bifurcated H-bond from Arg301 to the R–OH of D-lactate may account for its orientation and nucleophile activation. This orientation is observed when the guanidino side chain of this residue is flexible. D-Alanine adopts an orientation that utilizes H-bonding to water 2882 and the D-alanyl phosphate in subsite 1. Both of these orientations provide mechanisms of deprotonation and place the nucleophile within 3.2 Å of the electrophilic carbonyl of the D-alanyl phosphate intermediate for formation of the transition state.

These results suggest that Arg301 has a dual function in a sequential reaction mechanism, i.e. substrate orientation in subsite 2 as well as stabilization of the transition state. In addition, these docking studies provide insights for inhibitor design targeted to this subsite of the ligase.

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1. Introduction

D-Alanyl-D-alanine is an essential intermediate in the biosynthesis of peptidoglycan, a major structural polymer of the bacterial cell wall. The synthesis of this dipeptide is catalyzed by D-alanine:D-alanine ligase (ADP) [1–3]. Phylogenetic analyses of this enzyme define five subfamilies [4–6]. One of these, represented by *Leuconostoc mesenteroides*, also utilizes D-lactate as a substrate together with D-alanine in this ligation [7]. Thus, the organism can synthesize cross-linked peptidoglycan using either UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala or UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-lactate [7–9].

Kinetic analyses of the D-alanine:D-alanine ligase family established two subsites in the catalytic center for binding D-alanine [10–12]. Subsite 1 is characterized by both a higher affinity and a higher specificity for D-alanine than subsite 2 [10,13]. For example, with the ligase from *Enterococcus hirae* (formerly *Streptococcus faecalis R* 8043) D-α-amino-*n*-butyrate is a poor substrate for subsite 1 and a good substrate for subsite 2. Simultaneous incubation of these amino acids results in a significant synthesis of the mixed dipeptide (D-ala-D-α-amino-*n*-butyrate) as well as D-ala-D-ala [10]. The ligase from *L. mesenteroides* catalyzes the synthesis of the depsipeptide (D-ala-D-lactate) in addition to D-ala-D-ala when D-alanine and D-lactate are incubated simultaneously [7]. In each of the D-alanine:D-alanine ligases the catalytic events in subsite 1 culminate in the transfer of the γ-phosphoryl of ATP to the carboxyl group of D-alanine with formation of the aminoacyl phosphate intermediate in the presence of Mg^{2+} [12]. The orientations of D-lactate and D-alanine in subsite 2 of the ligase that result in both nucleophile generation and subsequent attack on the electrophilic center of D-alanyl phosphate in subsite 1 are not known.

From a combination of mutagenesis experiments [5,7,14] sequence alignments [4,6,15] and crystallographic studies [15–20] of ligases from a variety of bacteria, many of the amino acid residues that play primary role(s) in subsites 1 and 2 have been defined [14,15,21]. Kuzin et al. [17] established the crystallographic structure of the *L. mesenteroides* ligase with a transition-

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state analog in the presence of Mg^{2+} and ADP. From this structure amino acid residues in subsite 2 for ligand binding were identified. These include Arg301 located at the interface of the subsites functioning together with Gly322 to form the oxyanion hole for stabilization of the transition-state intermediate. In addition, four other residues in this ligase, Met326, Ser327, Leu328 and Phe261 determine in part the binding specificity of subsite 2 [6,17]. Ideally, one could establish the steric constraints and substrate orientations of D-alanine or D-lactate if it were possible to co-crystallize the ligase with either substrate. Since this has not been feasible, the binding modes and catalytic processes of D-lactate and D-alanine utilization in subsite 2 have not been readily elucidated.

Inhibition of peptidoglycan assembly by vancomycin results from complex formation of this glycopeptide antibiotic with the acyl-D-alanyl-D-alanine of the undecaprenol-diphosphate MurNAc-pentapeptide intermediate [22–24]. This complex prevents further peptidoglycan synthesis and crosslinking. With UDP-MurNAc-tetrapeptide as substrate no inhibition of glycan synthesis by vancomycin is observed [23]. Replacement of the acyl-D-ala-D-ala of UDP-MurNAc-pentapeptide with D-ala-D-lactate yields UDP-MurNAc-tetrapeptide-D-lactate. This analog substrate is effectively utilized for cross-linked peptidoglycan synthesis in the presence of vancomycin and, thus, resistance to the antibiotic results. Vancomycin 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 [22]. D-Alanine:D-alanine (D-lactate) ligases from *L. mesenteroides*, lactobacilli, and enterococci (VanA and VanB) that can synthesize D-ala-D-lactate, as well as D-ala-D-ala, are of major interest for their role in determining this resistance phenotype [5,6,17,19,25–28] In addition to the role of this ligase in vancomycin resistance, the enzyme has also been the focus of inhibitor design and screening programs to identify new antibacterial agents [15,29–33]. One of these programs [29] that utilizes AutoDock 4 for virtual screening has identified three promising inhibitors from the National Cancer Institute Diversity Set.

It is the goal of this paper to utilize AutoDock 4 to investigate the docking orientations of D-lactate and D-alanine in subsite 2 of the D-alanine:D-alanine (D-lactate) ligase from *L. mesenteriodes* and to study the role of Arg301 in assisting nucleophile activation in the ligation reaction.

2. Methodology

2.1. Software and PDB files

To examine the substrate orientations of D-alanine and D-lactate in subsite 2 of the ligase, AutoDock 4 and AutoDockTools 4 (ADT) were chosen [34,35]. The choice of AutoDock 4 for these studies was based on selective receptor flexibility in subsite 2 as well as flexible bonds in the ligands, D-alanine and D-lactate. A crystal structure [17] of D-alanine:D-alanine (D-lactate) ligase (ADP) from *L. mesenteroides* (PDB ID: 1EHI) was retrieved from the RCSB Protein Data Bank.

2.2. Ligand and protein files

The ligands, D-lactate, L-lactate, D-alanine and L-alanine, were drawn in ChemDraw and then converted to the AutoDock 4 PDBQT file format using ADT. In the case of alanine the zwitterion was chosen as the ligand [36]. The bonds of lactate (C^{α}–OH and C^{α}–COO^{-}) and of alanine (C^{α}–NH_3^{+} and C^{α}–COO^{-}) were allowed to rotate in the docking studies. This flexibility was also programmed into the PDBQT files with the use of ADT.

The PDB file for the D-alanine:D-alanine ligase contains the coordinates for monomers A and B of the unit cell. 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 in monomer A to the phosphinic acid **1** yielding the transition-state analog, phosphorylated phosphinate **2** (Fig. 1). Monomer A exists in a closed form restricting access to the catalytic center while monomer B, which lacks the phosphorylated phosphinate **2**, is in an open form [17]. Monomer A was energy minimized in Swiss-PdbViewer [37] before the following changes were made. Three modifications were made to the PDB file of monomer A. First, to provide access to subsite 2, the Ω-loop (residues 247–267) and the serine–serine loop (residues 185–188) were removed. Second, Kuzin et al. [17] observed that a water bridges between the carboxylate of phosphorylated phosphinate **2** and the backbone amide of Leu328 (Fig. 1). After removal of monomer B from the PDB file the bridging water is renumbered 2882. All other water molecules have been removed for these studies. Third, docking in subsite 2 utilizes enzyme-bound D-alanyl phosphate in subsite 1. To incorporate this structure, the phosphorylated phosphinate **2** in the PDB file was replaced with D-alanyl phosphate. In addition, the molecular charge on each of the two Mg^{2+} cations was varied between +0.8 and +1.2 and set at +1 according to Chen et al. [38] The molecular charges on ADP were calculated by the method of Gasteiger and Marsili [39]. Each of these modifications and calculations is essential for the docking studies in subsite 2. Using monomer A has the advantage of preserving the catalytic architecture of the amino acid side residues that determine subsites 1 and 2.

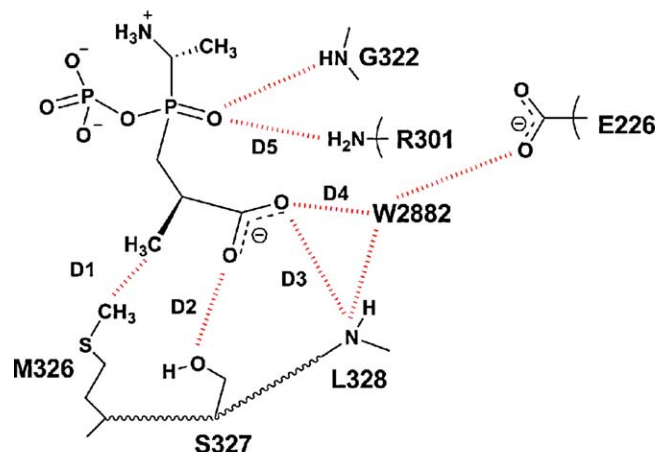

Fig. 1. Schematic identifying the distances of the transition-state analog, phosphoryl-phosphinate **2**, to key residues of the ligase listed in Table 1. The distances, D1–5 are designated. Gly322 and Arg301 determine the oxyanion hole (adapted from Ref. [17]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.3. Docking protocol

The basic docking protocol was adopted using the default settings provided by ADT [40]. The protein structure and the flexible Arg301 guanidino group were converted to PDBQT format in ADT. The Lamarckian Genetic Algorithm was used with a population size of 150 dockings. Five million energy evaluations were used in most dockings except where noted. All other parameters, e.g. crossover rate and mutation rate, were run with default settings. The grid size for specifying the search space was set at $21 \times 21 \times 21$ centered in subsite 2 with a default grid point spacing of 0.375 Å. The force field for AutoDock 4 was calibrated with a set of 188 protein–ligand complexes [34]. AutoDock 4 was launched in a Cygwin interface in the Windows operating system or from ADT in the Linux operating system. Docking logs were analyzed in the graphical user interface of ADT according to the

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