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Three-Dimensional Modeling and Analysis of the Peptidoglycan Connecting Peptide Bridge Synthesizing D-Alanine D-Alanine Ligase (Ddl) of Enterococcus Faecalis

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ABSTRACT

D-alanine: D-alanine ligase (DDL) is a vital enzyme in the bacterial cell wall biosynthesis. It is involved in the synthesis of D-alanine dipeptide which crosslinks the peptidoglycan for integrity. In this study, the 3-Dimensional structure of theDDL of Enterococcus faecalis (E.faecalis) was determined through Homology Modeling, on the basis of crystal structure coordinates of DDL of Staphylococcus aureus (PDB ID=2I80). Using a variety of programs the structure was then refined and analyzed for its active sites, ligand binding pockets, topology, conserved residues, and evolutionary relationship. The predicted model was then subjected to docking with norleucinephosphonate. It is concluded that structure of DDL-E.faecalis was quite similar to the DDL-S.aureus except the ω-loop, involved in substrate recognition/ binding, is present in the target molecule but missing in the template.

KEYWORDS: D-alanine: D-alanine ligase; Homology modeling; topology; Three-Dimensional structure; Enterococcus faecalis

1 INTRODUCTION

Bacterial cell wall is important for its survival and therefore many antibiotics are designed to target the cell wall of pathogenic bacteria. The cell wall of bacteria is majorly composed of peptidoglycan [1]. One of the enzymes in the biosynthetic pathway of peptidoglycan is D-alanine D-alanine ligase (DDL). DDL (EC 6.3.2.4) is of prime importance and it is a highly valued target now a day for the development of new drugs. DDL catalyzes the formation of D-alanine D-alanine dipeptide which further takes part in the crosslinking of the glycan chains in the peptidoglycan [2].ATP is utilized in this reaction. DDL's N-terminal binds the substrate while C-terminal is responsible for catalytic activity [3].

Enterococcus faecalis (E.faecalis) a gram positive, facultative anaerobe, non-motile, catalase negative [4], spherical in shape (occurring singly, as pairs or beaded chains) [5] commensal bacterium found in the GIT of humans and mammals [6,7]. It can resistant harsh conditions like temperature upto 60° C, pH highly alkaline upto 9.6, desiccants, draughts, chemicals (including alcohol, azide, salts, detergents, bile salts, heavy metals etc.). It can utilize a variety of materials including carbohydrates, many α-ketoacids, malate, citrateapart from glucose which is the main energy source for its metabolism [8].E.faecalis is known for causing nosocomial infections worldwide [9]. Other infections caused by E.faecalis are Bacteremia, Enterococcal endocarditis, Urinary tract infections (UTIs), meningitis, kidney infections, root canal infections, wound infections, respiratory tract infections (in rare cases) and other post- surgical infections [10, 11].The factors responsible for the high pathogenicity of E.faecalis are ability to survive in harsh environmental conditions [8], ability to utilize variety of energy sources[8], ability to form biofilms (e.g., on surgical instruments [12], medical devices, uteral stents [13], silicone gastrotomic devices [14], ocular lens materials, intravascular catheters and other instruments)[15, 16], cytolysin toxin, an aggregation factor encoded by its plasmid [17, 8], its ability to acquire resistance [18]. Multi-drug resistance [19] (semisynthetic penicillins, trimethoprim-sulfamethoxazole, cephalosporins, aminoglycosides, aztreonam, nafcillin, clindamycin and oxacillin and even vancomycin) [20, 21, 22].

2.METHODOLOGY

2.1. Primary Sequence Retrieval: The primary sequence of the target (DDL of E.faecalis) (DDL_ENTFA), Uniprot ID= Q47758) was retrieved from the Expert Protein Analysis System (ExPASy) [23]via the URL www.expasy.org.

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2.2. Alignment of Template and Target: The target sequence was run on BLAST (Basic Local Alignment Search Tool) program [24]. On the basis of BLAST results DDL of S.aureus (Protein Data Bank (PDB) ID= 2I80) was chosen as the template (homologous structure with known 3-D structure) whose sequence was retrieved from the SwissProt [25] and using the software BioEdit the template and target sequences were aligned.
2.3. Model Construction: Thirty energy minimized models were made using the software MODELLLER 9v7 [26] on the basis of the similarity between the template and target.
2.4. Model Evaluation: These thirty models were then evaluated through ProCheck program [27] and on the basis of results from ProCheck, one best target model was chosen out of those thirty models and it was our required target model. Energy check was then done for that model through online ProSA software [28].
2.5. Visualization: Then the model was visualized through Discovery Viewer (DS) v3.5 software [29] for its active sites, ligand binding sites, overall structure, important domains and its different types of interactions with the ligand molecule. Superposition of the target and template structures was done through DS viewer v3.5 software. Ligplot construction was done using the Profunc server [30] which was accessed via the link ebi.ac.uk/thornton-srv/databases/profun/.
2.6. Multiple Sequence Alignment: Multiple Sequence Alignment (MSA)was done for the target model DDL- E.faecalis with 10 representative species of the DDL family through BioEdit software and CLC Bio main Workbench 6.9. Phylogenetic tree was constructed for them using the CLC Bio main Workbench v6.9 software.
2.7. Docking: Finally docking for the target model DDL of E.faecalis was done with the Phosphonate inhibitor in this particular study through Patch dock online server[31]and the resultant best solution was visualized, studied and analyzed with DS viewer v3.5.

3. **RESULTS AND DISCUSSION**

BLAST results showed that both the sequences have 167 identical residues out of 346 (48%), 239 positives or similar residues out of 346 (69%). Both the sequences have 874 BLAST bit score between them. E-value is 1e-114 and 5 gaps out of 346 (1%). BLAST results showed that the DDL of S.aureus (PDB ID= 2I80) is homologous to the target sequence.
3.1. Overall 3-D Structure: The overall structure of the DDL- E.faecalisis quite similar to the DDL-S.aureus with α/β/α topology and can be divided into three major domains (fig.1.b.), domain 1 consists of residues from N-terminus M1-L124 which includes 4 α-helices(α1-4 and a portion of 5th alpha helix) and 5 β-sheets (β-1-5). Domain 2 consists of residues from A125-E222 which includes 5 α-helices (α5-9)and 4 β-sheets(β6-9) and a part of the beta sheet 10). Domain 3 consists of residues I223 – K348 of the C-terminus which includes 3 α-helices (α10-12) and6 β-sheets; β10-15) and a ω-loop consisting residues Y248-H273 as shown in fig.1.b and fig 8.

The DDL of S.aureus3-D structure also consists of three domains with alpha/beta topology (fig.1.a.). The ω-loop region however which plays an important role in substrate recognition and binding is missing and disordered in the template inhibitor bind structure [3] but this loop is in ordered position in the phosphinate and ADP.Mg^{2+} complexed structure of DDL. Also the ω-loop is not missing or disordered in the target model. From this information it could be proposed that if this loop plays important role in substrate recognition and binding it may also play an important role in the target model or may be play the same role.

Fig.1.(a) Overall 3-D structure of the DDL- S.aureus(b). Overall 3-D structure of the DDL- E.faecalis. Domain 1, 2, 3 and ω-loop region colored in magenta, yellow, blue and red respectively. Ligand GIL349 in stick model (green color) present at the interface of first and third domain, shown and labeled. The N and C terminals are also shown. Display stype= solid ribbon. Image generated by DS visualizer v3.5 software.+
3.2. Conserved residues of Target and Template: The conserved residues in both target and template which are:
In the domain 1 of the target most conserved residues consists of "24 VLNAI 28", "97 LHGPNGEDGTIQG109","117 PYVG 120". While the conserved residues include "62 LHL 64","93 VFP 95" and "122 GVL 124".In domain 2, most conserved residues consists of "174 YPVFVKPAN182" and "184 GSSVGISK 191".While the conserved residues includes "130 MDK 132", "153 LRS 155" , "204 EAF 206" and"215 EQG 217". In domain 3, most conserved residues consists of "220 AREIEVA 226", "238 PGEVVKDVAF 247", '262 QIPA 265", "312 MPGFT 316", "318 FSMYP 322" and "324

LWENMGL 330.While the conserved residues includes,"228 LGN 230","289 SGL 291"and "295 DFF 297".

3.3. Procheck and ProSA Results:

Model	Core	Allowed	Generously	Allowed	Disallowed	Z-score (Threshold 0.0)
Template	2I80	91.8%	7.2%	0.3%	0.7%	-8.56
Target Efe26	90.1%	7.9%	1.3%	0.7%	-8.7	

Table 1.Ramachandran plot statistics obtained as results of Procheck and Z-scores energy for the template and target models from ProSA analysis.

The results from procheck revealed that DDL of E.faecalis has total 348 residues constituted the model, among which 274 residues (90.1%) were in the most favoured region, 24 residues (7.9%) in the allowed region, 4 residues (1.3%) in the generously allowed region and 2 residues (0.7%) in the disallowed region as shown in table 1 and fig.2. Table 1 also shows the Z-score obtained from ProSA analysis which are way below zero and indicate the low energy of the model and hence more stability.

Fig.2. Ramachandran plot of the DDL- E.faecalis obtained from Procheck.

3.4. Superposition Result: Most of the regions of target structure (DDL- E.faecalis) superpose with the template (DDL- S.aureus), only few regions of target and template were clearly and fully non-superposed. D244-E260 residues in the target were fully non-superposed and most of the residues of template in this region were missing because most of this region comprises the ω-loop region which plays role in substrate recognition and binding and this ω- loop is missing in template. Other fully non-superposed area of target is S41-D43. The template has region D242-F245 which is non-superposed with the target. Residues Q346-S358 at the C-terminal region are present in template but are absent in the target (fig.3).

Fig.3. Superposition image of the DDL – E.faecalis with DDL- S.aureus.(a). Non-superposed regions of the target protein are labeled (b). Side view of the superposed target-template with non-superposed and excess residues of the template labeled. Display style solid ribbon style. Target= cyan ribbon, template=red ribbon. Ligand molecules shown in stick display where target ligand= pink, template ligand= green.

3.5. Evolutionary Relationship: The phylogenetic tree (fig.4) results showed that the E.faecalis has close evolutionary relationship with the S.pneumonia and S.aureus (template) which means that they might have somewhat similarity in their structure and thus functions. It shows that S.aureus and E.faecalis had a common ancestor in the distant past.

Fig.4. Phylogenetic tree showing the template S.aureus and the target E.faecalis and their evolutionary relationship with members of DDL family.

3.6. Ligand Binding Site: The ligand binding pocket was revealed by target protein visualization in the DS viewer software. In the ligand G1L349 (chloro dimethyl tri-fluoropropanamide) molecule, the chloro dimethyl constitutes the head while the tri-flouropropanamide constitutes the bottom of the ligand GIL349. The residues at the top of the head of ligand are S17, S20, V16. The residues H98, L97 and V96 are side wise towards the head side. While L330, L291, V119, G120, F94 and P95 constitute the bottom of the ligand binding pocket. HOH 363 forms H-bond (bond distance=2.93 A°) with the F22 of the ligand GIL349. F315 and P313 face the ligand side wise. The ligand GIL349 binding site is hydrophobic mainly and present at the interface of the first and the third domain (fig.5.a.)

3.7. Identification of Active Site:

Active site residues of the template 2I80 were analyzed by DS viewer includes S20, T23, F92, P93, L94, L95, H96, V117, G118, L289, M310, P311, G312 and HOH414.The active site residues of the target analyzed by DS viewer are S20, S23, I92, V93, F94, P95, V96, P117, Y118, S289, N310, T311, M312 and a water molecule HOH 363 (fig.5.b.).

Fig.5. (a).shows Line display of ligand binding pocket of target with ligand GIL349 (green colored in stick display). (b). Showsprotein's active site residues, showing interaction of ligand GIL349 (green color) with water at bond distance of 2.937 A° which in turn has an H-bond (red color) with other residues. DS viewer v3.5 used for generation ofthis image.

3.8. Ligplot results: In the Ligplot result of the target, oxygen atom attached with the alpha carbon of Pro 313 of the target form H-bond with the N11 of the ligand G1L 349 (bond distance 2.96 A°) and these residues V119, L97, S20, H98, M312, P95, V96, V16, F315 and G314 have hydrophobic contacts with the ligand GIL349. While the Ligplot of the template showed that oxygen atom of P311 of the template form hydrogen bond with the N11 of the ligand G1L 400 (bond distance 2.95 A°) and these residues F313, L289, V117, P93, L94, L95, M310, H96 and G312 of target have hydrophobic contacts with ligand 349(fig.6).

Fig.6. Pro313 (A) of E.faecalis having hydrophobic contacts with Gly 314, Phe315, Val 16, Ser 20, His 98, Met 312, Val 119, Val 96, Pro95, Leu97 (constructed via Ligplot) and the oxygen atom of the Pro 313 of the target molecule form bond with the N-11 of the ligand GIL349 with a bond distance of 2.96 A°. Atoms shown in colors, Carbon (black), Nitrogen (blue), Oxygen (red), Chlorine and Fluorine (green).

3.9. Surface Interactions Among Model Protein and Ligand: Regarding Hydrogen bond (H-bond) surface interactions, F22 (fluorine 22) of ligand GIL349H-bonded with Oxygen of HOH 363 (2.937Å°). N (nitrogen) of F315 formed H-bond with O (oxygen) of HOH369 (2.836), N of L97 H-bonded with O of P95 (3.087), OG (gamma) of S20 H-bonded with O of S17 (2.601), OG of S20 H-bonded with O of V16 (3.160) and OG of S17 H-bonded with O of V96. Regarding Aromatic surface interactions, F 94, P95, V96, L97, S17 and L339 interact edge wise P313, F315 and H98 interact side wise with the ligand GIL349. L291, L339, V119, F94,P95, P373, V96 had hydrophobic surface interactions with the ligand at the bottom side of ligand GIL349 while only H98 was giving a slight hydrophilic effect at the entrance or head side of ligand pocket. No noticeable electrostatic surface interactions were present (fig.7.)

Fig.7. Surface interactions between ligand GIL349 molecule (shown inside the pocket in light green color) and the residues of the DDL- E.faecalis(a).Shows H-bond surface interactions, (b). Shows face and edge wise interaction, (c). shows Hydrophobic interactions and (d). shows electrostatic interactions.

3.10. Topology of the Modeled Structure: The target protein D-alanine D-alanine ligase of E.faecalis has overall α/β/α topology. According to the PDB sum server analysis target protein has 12 helices, 10 helix-helix interactions, 24 beta turns, 1 gamma turn, 3 beta sheets, 1 beta-alpha-beta unit, 6 beta hairpins, 3 beta bulges and 15 beta strands (fig.8).

Fig.8. Topology of the DDL- E.fecalis H1-H12 represents α-helices and arrows represents 15 β-sheets.

3.11. Docking Results: Results from docking shows that phosphonate inhibitor Norleucine phosphate selected for this study interacts such that the N (nitrogen) of the inhibitor form H-bond (2.605A°) with Oxygen (OE1) of E 308 of target. Also Oxygen atom of the inhibitor form H-bond (3.137A°) with the Nitrogen of K132 which in turn forms H-bonds with Oxygen (OD1) of D131 (2.995A°) and Oxygen (O) of A121 (3.045) (fig.9).

Fig.9.Interaction of Phosphonate inhibitor Norleucine phosphate with the residues (labeled with single letter amino acid code and number) in the DDL of E.faecalis.The phosphonate inhibitor shown in bright green color. Image generated by DS viewer v3.5program.

4. CONCLUSION

The DDL model based on template matched in many aspects. The overall quality of the model is good. The model varies from its template in a few regions because the sequence identity is 48%. The result from ramachandran plot is good. It shows more than 90% residues in the most favoured region. Energy wise the model showed stability. Had a good Z-score of -8.75 which is even better than template -8.5. The active site residues of the model are all conserved according to the multiple sequence alignment and evolutionary relation wise the model and S.pneumonia DDL has branched from same ancestor in the near past while it shared the same ancestor with the S.aureus DDL in the distant past. Docking was done to study the interaction and orientation of the ligand (inhibitor) drugs with the target protein model to know the potential and possible action of the drug on the target protein to treat infections caused by multidrug resistant life threatening Enterococcus faecalis.

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