Original Research Paper

Probing the Substrate Specificity and Intersubunit Interactions of *Brevundimonas Diminuta* Glutaryl Acylase with Site-Directed Mutagenesis

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Abstract: Glutaryl-7-ACA acylases (GLA) are industrial enzymes widely used for the production of 7-aminocepahlosporanic acid (7-ACA)-the starting compound for manufacturing of semisynthetic cephalosporin antibiotics. Generation of mutant GLA`s with increased activity and stability, capability for single-step conversion of cephalosporin C (CPC) directly to 7-ACA is a promising route to improve the current biocatalytic technologies. In this study GLA from *Brevundimonas diminuta* (BrdGLA) has been rationally re-designed to produce enzyme variants with improved properties. First, sequence analysis was performed to select residues responsible for substrate specificity as hotspots to introduce the capability to bind CPC in the active site of BrdGLA. Molecular modeling was used to evaluate the influence of selected residues on the formation of productive enzyme-substrate complex and the catalytic conversion. Genes, encoding mutant enzymes, were constructed, expressed and recombinant enzymes were purified on the chitin affinity resin. The mutant proteins showed induced catalytic activity against CPC. Second, BrdGLA mutants with increased activity and stability in alkaline conditions were obtained by mutating one of the surface lysine residues and replacement of the glutamine residue located in the active center by asparagine. Finally, structural analysis was used to select amino acid residues involved in formation of the quaternary structure of BrdGLA. Replacement of these "interface" positions to alanines led to a significant enzyme destabilization and reduction of its activity, confirming the role of the identified residues in the intersubunit interactions. The glutaraldehyde cross-linking has shown that the wild-type enzyme and its "interface" mutants possess complex oligomeric structure in solution with predominance of tetrameric forms.

Keywords: Cephalosporin C, Biocatalysis, Heterologous Expression, Molecular Modeling, Site-Directed Mutagenesis

Introduction

Acylases of glutaryl-7-aminocephalosporanic acid (Gl7ACA-acylase, EC 3.5.1.93, hereafter-glutaryl-acylases, GLA) are industrial enzymes extensively used for the production of 7-aminocephalosporanic acid (7-ACA)-the starting compound for manufacturing of semisynthetic cephalosporin antibiotics. Conversion of Cephalosporin C (CPC) to 7-ACA may be carried out either by chemical hydrolysis, requiring the use of highly toxic compounds and special reaction conditions at very low temperatures (Fechtig *et al*., 1968), or by enzymatic conversion (Sonawane, 2006). Currently known reactions of enzymatic CPC transformation can be divided into two groups: (i) Single-step conversion, using, for example, cephalosporin

C acylase from *Pseudomonas* (Conti *et al*., 2014) and (ii) two-step conversion, that implies the conversion of CPC to glutaryl-7-ACA using a D-amino acid oxidase (DAO; EC 1.4.3.3) from *Trigonopsis variabilis* (Szwajcer-Dey *et al*., 1990) or *Rodotorula gracilis* (Pollegioni *et al*., 1997) followed by GLA-catalyzed hydrolysis of the glutaryl-7-ACA to form 7-ACA and glutarate (Conlon *et al*., 1995) or by enzymes of other classes belonging to the gamma-glutamyltransferase (Yamada *et al*., 2008).

GLAs are of the most significant practical interest for biocatalytic synthesis of 7-ACA. GLAs are related to penicillin acylases. The latter are widely used in the industrial production of semisynthetic beta-lactam antibiotics (Srirangan *et al*., 2013). Similar to PAs, GLAs consist of two different subunits-α and β, but the

three-dimensional structures of enzymes are different in the two families. PAs are heterodimeric whereas GLAs are heterotetramers composed of two heterodimers, which are formed from a single-stranded polypeptide precursor resulting from the-pecific processing. GLAs can effectively hydrolyze glutaryl-7-ACA, however cannot convert CPC. The exceptionally high biotechnological value of GLAs dictate the interest in the development of effective and efficient systems for production of these enzymes. We have previously developed efficient expression system for production of *Brevundimonas diminuta* GLA as a fusion with N-terminal chitin binding domain (BrdGLA/ChBD), enabling single-step purification of hybrid enzyme on the chitin-column (Khatuntseva *et al.*, 2008). We have also shown that this modification does not affect protein activity and processing of GLA precursor and is even beneficial for protein thermal stability and catalytic properties. The developed expression/purification system provides an attractive opportunity for creation of novel GLA analogs to overcome several drawbacks of existing biocatalytic technologies. These drawbacks include, but are not limited to relatively low specific enzyme activity, its instability under the influence of mild denaturing agents and "crosslinking" agents, acidic and alkaline pH etc. (Monti *et al.*, 2000).

The 3D structures of GLA precursor and mature processed form have been established (Kim *et al.*, 2000), as well as the complex of this enzyme with the substrate and reaction products (Kim and Hol, 2001). These studies revealed important details of the structure of GLA active site, the mechanism of enzymatic reaction as well as autocatalytic enzyme processing and have opened the way to obtain various GLA analogs using methods of protein engineering. The main achievements in GLA protein engineering so far have been related to application of directed evolution to obtain variants with altered substrate specificity and site-directed mutagenesis of surface residues. Such variants are promising biocatalysts for production of 7-aminodesacetoxycephalosporanic acid, an important intermediate in the synthesis of semisynthetic oral cephalosporins (Otten *et al.*, 2002), and for development of more efficient methods of enzyme immobilization on various polymer carriers (Zhang *et al.*, 2005).

Studies devoted to investigation of GLA quaternary structure are rather limited. GLA quaternary structure was reported to be rather labile (Battistel *et al.*, 1998) and easily distorted by application of mild denaturants and extreme pH values. Generation of GLA analogs with more tight intersubunit interaction is a promising route towards enzyme stabilization. Other important unsolved issues are the identification of amino acid residues determining GLA substrate specificity, elucidation of enzyme kinetics and study of intersubunit interaction.

In this study GLA from *Brevundimonas diminuta* (BrdGLA) has been rationally re-designed to produce enzyme variants with improved properties. By using sequence and structure analysis we have obtained more active and stable GLA analogs as well as GLA variants capable for single-step conversion of CPC to 7-ACA. Furthermore, electrostatic interactions in the interface between the heterodimers were studied to evaluate the role of different amino acid residues in formation of the quaternary structure.

Materials and Methods

Bacterial Strains and Plasmids

Escherichia coli XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIqZΔM15 Tn10 (Tetr)] (Stratagene, USA) was the host strain for the construction of BrdGLA/ChBD mutants. Mutant enzymes were expressed in *E. coli* BL21 (DE3) [F-ompThsdSB (rB- mB-) dcm gal dcm (DE3). The vector for expression of constructed BrdGLA/ChBD variants was pSVH0108, containing the gene for BrdGLA/ChBD fusion protein under the control of T7 promoter and terminator (Khatuntseva *et al.*, 2010).

DNA Manipulations

Isolation of plasmid DNA, DNA hydrolysis with restriction endonucleases, isolation of DNA fragments from agarose gels, ligation of DNA fragments, PCR amplification was performed using enzymes and kits produced by "Sib Enzyme" (Russia), "Eurogen" (Russia) or "Fermentas MBI" (Lithuania)in accordance with the manufacturer's recommendations. DNA sequencing was performed using ABI BigDye3.1 sequencing kit (Applied Biosystems) according to the manufacturer's protocol on ABI3730 DNA sequencer. Site-directed mutagenesis was carried out using Quick-change site-directed mutagenesis kit (Stratagene, USA). After final digestion with DpnI the reaction mixture was purified using CleanUp Mini kit (Eurogen, USA), 5 µls out of 15 µL of purified sample were used to transform competent *E. coli* cells.

Expression and Purification of BrdGLA/ChBD Variants

E. coli BL21 (DE3) transformants, expressing various BrdGLA/ChBD variants were cultured in 100 mL of Overnight Express™ TB medium (Novagen, USA) with 50 µg/ml kanamycin in "auto induction" conditions for 36 hours at 28°C. Cells were collected and suspended in 10 mL of Buffer A (50 mM sodium phosphate, pH 7,5, 1 mM EDTA, 0.3% CTAB, 2% glycerol) and disrupted by sonication. Crude cell homogenates were centrifuged at 8000 g, 4°C for 20 min. Resultant supernatants were purified by 30 to 65%

saturation of ammonium sulfate at 4°C to get protein samples containing recombinant enzymes. Precipitates were dissolved in buffer B (50 mM sodium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, 2 mM EDTA, 10% glycerol, 2M NaCl) and applied to a column (1×3 cm) with chitin beads (New England Biolabs, USA) equilibrated with buffer B. The column was washed with the same buffer until the extinction of absorption at 280 nm. Proteins specifically bound to the chitin column were eluted with Buffer C (50 mM sodium phosphate, pH 7.5, 1 mM EDTA). The final enzyme preparations were concentrated by ultrafiltration on Amicon Ultra-15 centrifugal filter (Millipore) and assayed by SDS-PAGE. Protein concentration was determined by the method of (Bradford, 1976).

Denaturing Gel Electrophoresis

Electrophoresis under denaturing conditions was carried out according to the method of (Laemmli, 1970) using a 12% separating and 4% stacking polyacrylamide gels. For separation of protein samples cross linked with glutaraldehyde 7.5-20% denaturing gradient gel electrophoresis was used. The gel was poured so that the gel porosity increases from top to bottom.

Enzyme Assays

To determine the activity of BrdGLA/ChBD variants colorimetric method based on the reaction of chromophore formation upon interaction of 7-ACA amino group with p-dimethylbenzaldehyde was used as previously described (Khatuntseva *et al*., 2008). One unit of activity was determined as the amount of enzyme (in milligrams) required to convert 1 μmole of substrate during one minute of incubation. Specific activity was determined as the amount of enzyme (μmoles of substrate per min at 37°C) per mg of total protein in a sample. The total protein content was determined using method (Bradford, 1976).

Determination of BrdGLA Stability

To determine the stability of the wild-type enzyme and mutants thereof, samples with enzyme concentration of 43 μg/ml were incubated at 35ºC in 0.1 M K-phosphate buffer, pH 7.0-8.0 or 0.05 M K-phosphate buffer, pH 10.0. After distinct time intervals (from 1 hour to 3 days) enzyme aliquots were removed and activity was measured.

Crosslinking of Enzyme Subunits with Glutaraldehyde

Enzyme samples diluted in 50 mM borate buffer pH 9.5 to a final concentration of 300 μg/mL were mixed with 1/5 part of 1% aqueous solution of glutaraldehyde and the mixture was incubated for 40 min at 35°C. The reaction was stopped by adding Tris-glycine buffer (25 mMTris, 200mm glycine, pH 8.3). Samples were kept at room temperature for 5 min. Obtained samples were analyzed by electrophoresis under denaturing conditions.

Molecular Modelling of the Full-Size Structure of BrdGLA/ChBD Fusion Protein

Modelling of the full-size structure of *B. diminuta* GLA as a fusion protein with N-terminal chitin binding domain (BrdGLA/ChBD) was performed with Modeller v.9.12 (Šali and Blundell, 1993). High-resolution crystallographic structure of *B. diminuta* GLA in complex with glutaryl-7-aminocephalosporanic acid (PDB code 1JVZ) and the structure of ChBD domain from *B.circulans* chitinase A1 (PDB code 1ED7) were used as structural templates. The quality of the first 20 models was evaluated using the DOPE energy estimate, which is based on statistical comparison of obtained models with existing high-resolution crystallographic structures. The best candidate structure was selected and its geometry optimized by molecular modeling according to the protocols described earlier (Suplatov *et al*., 2014). The resulting structure contained BrdGLA with N-terminal chitin binding domain in complex with glutaryl-7-aminocephalosporanic acid. The distances and angles of the crucial catalytic interactions in the active site satisfied the knowledge-based criteria of amidase catalytic activity (Radisky and Koshland, 2002). This molecular model of the enzyme-substrate complex was further used for computational research.

Sequence and Structural Analysis of Substrate Specificity in BrdGLA

To select positions responsible for substrate specificity of BrdGLA the following procedure was used. First, all residues within 5Å from the acyl moiety of the substrate were selected. Among these, the following residues were not further considered: Serβ1-the catalytic nucleophile; Valβ70 and Asnβ244-oxyanion hole residues; Proβ22-because its mutation can compromise structural organization; Hisβ23 and Thrβ221-because their side-chains are not involved in binding of the acyl moiety of the substrate. The remaining 9 positions were considered as hotspots for mutagenesis to design substrate specificity of BrdGLA to CPC. Sequence analysis was used to predict the amino acid substitutions at each selected site. Pfam alignment of the Ntn-hydrolase super family of enzymes (PFAM code 01804) was filtered for non-redundancy at 90% pair wise sequence identity. The resulting multiple alignment contained 845 protein sequences. Distribution of amino acid residues in the selected hotspots was evaluated. The following strategies were used to choose the particular amino acid substitutions at each selected position. First, the consensus approach was implemented, which implies that the most frequently observed amino acid types are favored

by natural evolution and can be used to produce enzymes with improved properties (Lehmann *et al.*, 2002). Therefore, the most popular amino acid types at each position were selected as candidate substitutions. Secondly, if the charged residues were present in other homologs in a corresponding position of the multiple alignments they were included into the list of mutations to accommodate the D-α-amino adipyl moiety of CPC which contains both positively and negatively charged groups. Finally, mutation of large hydrophobic residues to smaller hydrophobic residues was considered to accommodate the larger acyl part of CPC. The following hotspots and mutations were selected (frequency of amino acid types in corresponding positions within the Ntn-hydrolase super family are shown in parenthesis): Yα149 → L(37%), F(12%), R(4%), D(1%), K(<1%), E(<1%); Lβ24 → R(10%), E(<1%); Fβ31 → S(28%), G(13%), V(3%), L(3%), R(2%), D(2%), E(1%), K(<1%); Yβ33 → W(49%), R(6%); Qβ50 → L(31%), F(24%), E(5%), R(<1%), H(<1%); Rβ57 → V(20%), L(16%), I(14%), H(5%), E(2%), R(<1%); Fβ58 → I(36%), L(18%), V(9%), H(1%), D(<1%), E(<1%); Nβ68 → H(18%), L(15%), R(<1%), E(<1%); Fβ177 → Q(32%), L(12%), V(11%), E(4%), H(<1%), D(<1%). The role of the selected residues was further evaluated by molecular modeling.

Molecular Modeling of Substrate Specificity in BrdGLA and Screening of in Silico library of Mutants

Molecular models of BrdGLA variants with substitutions suggested by sequence and structure analysis were generated and screened by molecular docking using CPC as the substrate. All single mutants, as well as all double and triple combinations of the selected mutations were considered. Protocols for molecular docking with the Auto dock software (Huey *et al.*, 2007) and preparation of *in* silico libraries were discussed previously (Suplatov *et al.*, 2012). Structural filtration of the resulting enzyme-substrate complexes was used as a post-docking tool to select mutants that were able to stabilize the productive binding state in the active site. Distances of crucial catalytic interactions that describe trajectory of the nucleophilic attack were restricted as follows. Distance between the carbonyl carbon atom of the substrate and the γ-oxygen catalytic Serβ1, as well as distances between the substrate`s carbonyl oxygen and main-chain of Valβ70 or side-chain of Asnβ244-were limited to 3.5 Å. In addition, the absolute values of angle and dihedral angle of a nucleophilic attack trajectory of the Serβ1γ-oxygen on 80 to 100° (Radisky and Koshland, 2002). Enzyme-substrate complexes that were out of range were dismissed. The final results were ranked by the frequency of the productive binding geometry (how many times the near-to-attack conformation was observed after 100 independent docking runs). The first top-ranked mutant was further evaluated experimentally. Scientific illustrations of biological macromolecules were prepared with PyMol software.

In Silico study of the BrdGLA Quaternary Structure

Crystal structure of a heterotetrameric structure of GLA from *Pseudomonas sp. 130* (PDB code 4E56) was used as a template to model the quaternary of BrdGLA/ChBD.

Results and Discussion

BrdGLA Variants with Altered Substrate Specificity

Structural analysis of the active site of BrdGLA in a complex with glutaryl-7-aminocephalosporanic acid suggested three sub-sites for binding of different functional moieties of the substrate: (1) The acyl moiety, which is the substituent at the C7 carbon atom of the β-lactam ring; (2) the β-lactam ring and (3) substituents at the six-membered cycle of the β-lactam ring. The most intense intermolecular interactions were observed in the binding site of the acyl moiety of the substrate: Tyrα149, Tyrβ33, Glnβ50, Argβ57 stabilize the negative charge on the carboxyl group, while Leuβ24, Pheβ58, Valβ70 and Pheβ177 form a hydrophobic environment for the atoms C9, C5 and C17 of the glutaryl moiety. These interactions determine specificity of the enzyme towards the native substrate (Fig. 1).

To extend the substrate specificity of BrdGLA we have re-designed the active site to accommodate the CPC. Compared to glutarylmoiety of Gl7ADCA, the D-α-amino-δ-adipyl moiety of CPC contains both positively and negatively charged groups and has a larger hydrophobic side-chain. Therefore, the active site should contain a positively charged amino acid residue for binding of the carboxyl group and also a negatively charged as partate or glutamate to stabilize the positively charged amino group. The corresponding *in silico* library of BrdGLA variants was generated and screened. Since the BrdGLA binding site lacs as partate or glutamate residues in the active site, the respective counter ion was introduced as well as the overall volume of the bind cleft was increased by substitutions to smaller hydrophobic residues. The triple mutant Yα149E/Fβ31L/Fβ177L was found to be the best in accommodating the proper binding and the near-to-attack conformation of the substrate in the active site. Structural analysis of the resulting enzyme-substrate complex showed that CPC binding at the active site of the mutant enzyme is similar to binding of Gl7ACA in the active site of the wild-type protein (Fig. 2). The acyl moiety binds inside a deep cone-shaped cavity which has charged and polar amino acid residues located at the bottom.
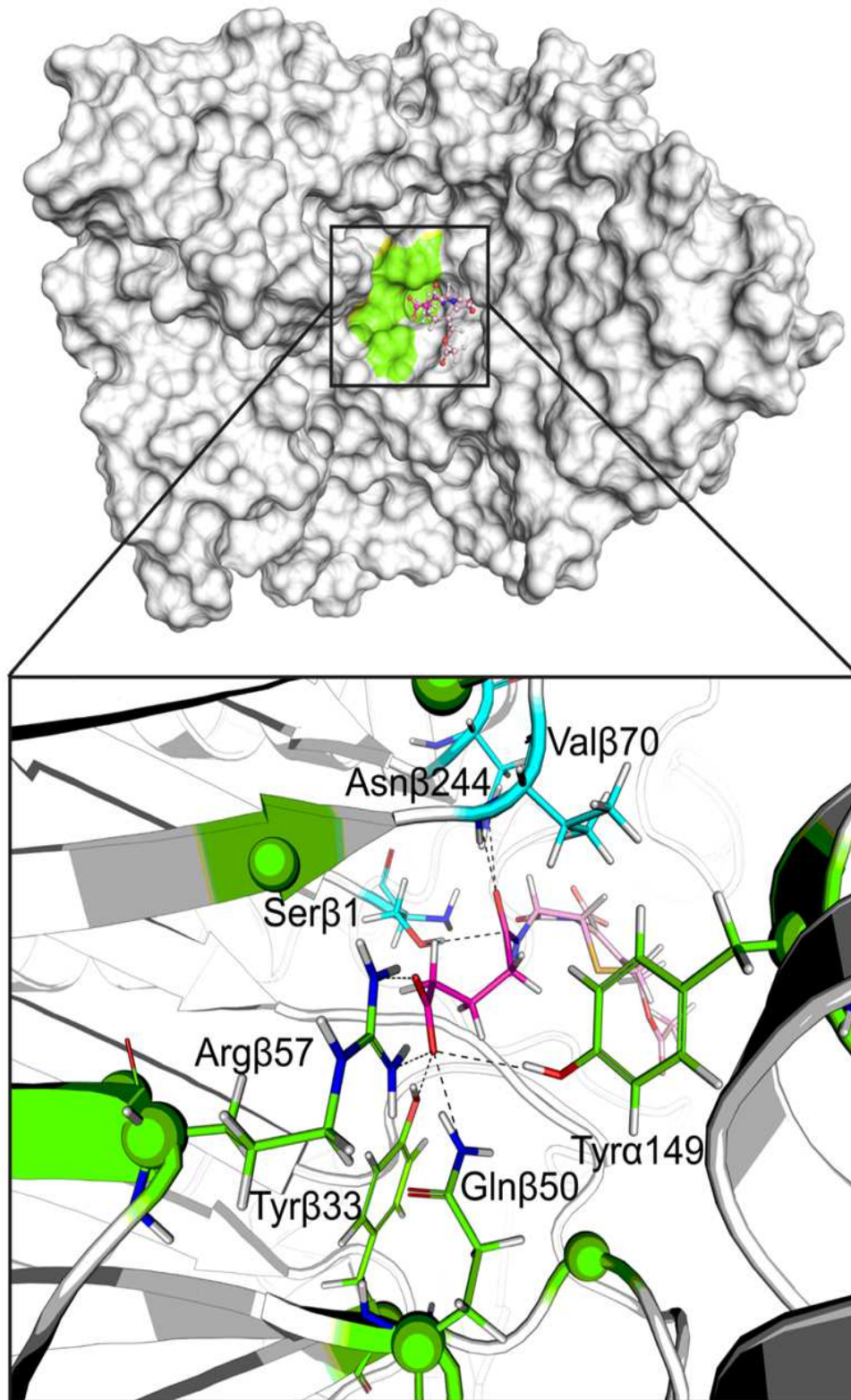
Fig. 1. BrdGLA in a complex with glutaryl-7-aminocephalosporanic acid. Glutaryl moiety of the substrate is colored in magenta. Dashed lines indicate electrostatic interactions between the active site residues and the substrate. Cα-atoms of residues selected as hotspots for mutagenesis are shown as green spheres
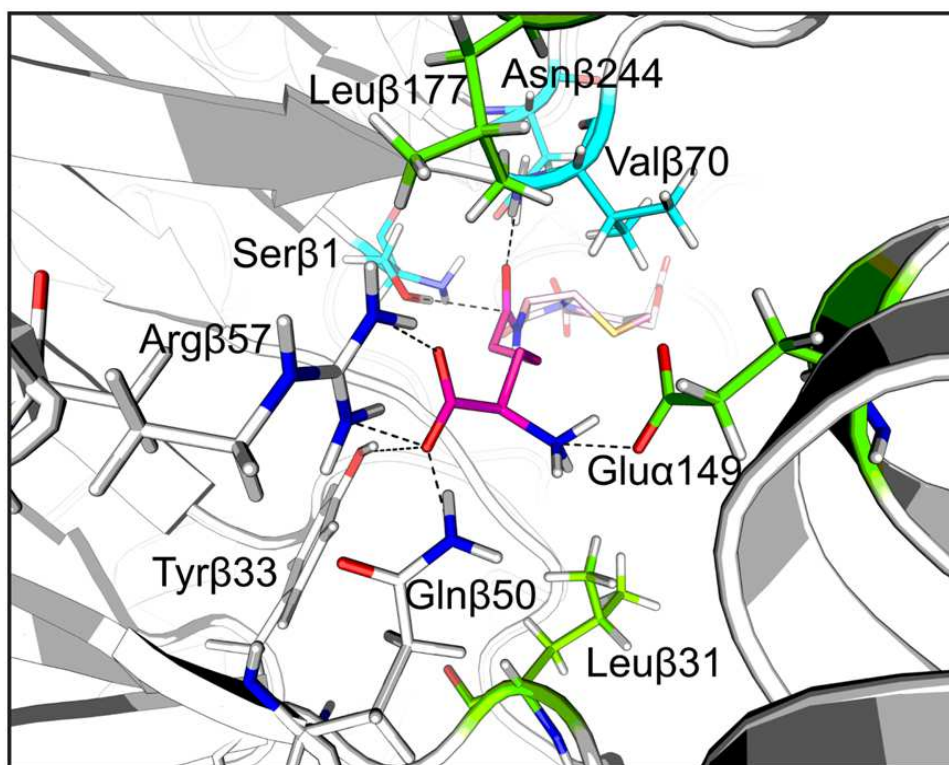
Fig. 2. BrdGLA variant Yα149E/Fβ31L/Fβ177L in a complex with cephalosporin C. D-α-amino adipyl moiety of CPC is colored in magenta. Residues introduced by point mutagenesis are colored in green. Dashed lines indicate electrostatic interactions between the active site residues and the substrate

Tyrβ33, Glnβ50 and Argβ57 residues interact with carboxylate of the D-α-amino-δ-adipyl moiety of the substrate, which adopts orientation similar to the carboxylate of the glutaryl moiety of the Gl7ADCA. At the same time, Yα149E accommodates the positively charged amino group of the CPC's acyl side-chain. The binding of the β-lactam ring, as well as its substituents occurs similar to Gl7ACA.

Based on the results of sequence and structure analysis the following single, double and triple BrdGLA variants were produced: Yα149E, Fβ177L, Yα149E/Fβ31L and Yα149E/Fβ31L/Fβ177L. Kinetic parameters of affinity-purified BrdGLA/ChBD variants were determined using Gl7ACA and CPC as substrates (Table 1). The highest activity towards Gl7ACA was detected for the wild-type enzyme. The triple mutant showed lowest activity with the native substrate. The activity of all enzyme variants towards CPC was improved compared to the wild-type enzyme; however appeared to be low in general. The activity of the triple mutant with CPC constituted not more than 0.3% of the activity of wild-type enzyme with the natural substrate Gl7ACA. The reason for such low performance can be explained by the inability of the mutant enzymes to hydrolyze CPC due to the problems with binding of the cephalosporin

nucleus. To overcome this limitation, additional efforts in re-designing the active site residues may be required. Nonetheless, obtained results show that the employed strategy can be used to extend substrate specificity of BrdGLA towards CPC.

When this article was in preparation we became aware of the very successful attempt to engineer GLA from *Pseudomonas* N176 towards mono-step process for CPC conversion (Conti *et al*., 2014). By combining structural knowledge, semi-rational design and evolution analysis authors were able to isolate enzyme variants with more than 11,000-fold increase in specificity constant and with the highest kinetic efficiency so far obtained for a cephalosporin acylase.

Site-directed Mutagenesis to Enhance BrdGLAStability and Activity

Previously it was shown that introduction of the double Kβ198A/Qβ50N mutation in *Pseudomonas* 130 GLA increases enzyme stability and activity (Zhang *et al*., 2005). We have introduced orthologous mutation in our BrdGLA/ChBD fusion protein and have studied properties of the mutant enzyme. Replacement of glutamine with asparagine at position 50 is expected to enlarge the substrate binding site and lead to stronger GL7ACA binding in the substrate pocket.

Table 1. Specific activity of BrdGLA mutants in enzymatic hydrolysis of GL7ACA and CPC

Enzyme	Gl7ACA		CPC
	Specific activity (μmole*min^{-1}*mg^{-1})	Compared to activity of wild type BrdGLA (%)	Specific activity (μmole*min^{-1}*mg^{-1})
BrdGLA wild type	8.750	100	0.0023
BrdGLA Fβ177L	0.770	8.8	0.0091
BrdGLA Yα149E	0.380	4.3	0.0069
BrdGLA Yα149E / Fβ31L	0.520	5.9	0.0110
BrdGLA Yα149E / Fβ31L / Fβ177L	0.045	0.5	0.0230
BrdGLAYα149E / Fβ31L / Fβ58L / Fβ177L	n.d.	n.d.	n.d.

Substitution of the surface lysine residue at position β198 with alanine should stabilize the tetramer structure. The activity of the double mutant measured in optimal conditions (35°C, pH 7.0) did not differ from wild type enzyme and constituted 7±0.5 μmoL*min^{-1}*mg^{-1}. The stability of the double-mutant at pH optimal for the wild type protein also did not increase: after 70 hours of incubation at 35°C and pH 7.0 the residual activity of the mutant protein was just 15% lower than the activity of the wild-type protein. Taking into account the reported influence of the Qβ50N mutation on pH-optimum of mutant enzyme and its stability at alkaline conditions (Zhang *et al*., 2005) we have studied the rate of thermal inactivation of the double mutant at pH 10.0. Results in comparison with previous experiment are shown on Fig. 3. It is obvious that both the mutant and the wild type enzyme are more stable at neutral pH (Fig. 3, lines 1 and 2) than at alkaline conditions (Fig. 3, lines 3 and 4). However, at pH 10.0 the mutant (Fig. 3, line 3) is 30% more stable than the wild type enzyme (Fig. 3, line 4) under the same conditions. Improved enzyme stability at alkaline pH accompanied with the high level of catalytic activity is favorable for industrial biocatalysis, since it overcomes partial enzymes inactivation that may occur at micro alkaline environment in the course of enzymatic 7-ACA production.

Investigation of BrdGLA oligomeric State and the Analysis of the Dimer-Dimer Interface

The data concerning the quaternary structure of GLAs is contradictory. According to the results of biochemical studies enzymes are tetramers (Battistel *et al*., 1998). At the same time, in the majority of publicly available 3D structures GLAs are represented mainly by α/β dimers, the fact that may be related to peculiarities of enzyme crystallization. In the recently obtained structures of unprocessed GLA precursor the protein is presented as a dimer of two fused α/β chains (PDB code 4E56). We have used this structure to study amino acid residues forming the interface between the two α/β dimers. Two clusters of intersubunit contacts were further studied experimentally.

Alpha-Subunit Mutants

Structural analysis revealed that amino acid residues Glnα91, Trpα92, Aspα120, Aspα121 and Ileα122 within the α-subunit participate in the formation of salt bridges, hydrogen and hydrophobic bonds between the two α/β dimers (Fig. 4).

To analyze the role of these residues the corresponding alanine mutants were produced - Qα91A/Wα92A, Dα121A/Iα122A, Dα120A/Dα121A/Iα122A. The mutant proteins were expressed in *E.coli* cells using the standard protocol and recombinant enzymes were affinity-purified on chitin sorbent. Kinetic parameters of purified protein preparations were determined using Gl7ACA as substrate. The highest activity was observed for the double Dα121A/Iα122A mutant (11 μmole*min^{-1}*mg^{-1}), while the activity of the triple Dα120A/Dα121A/Iα122A mutant was the lowest (2 μmole*min^{-1}*mg^{-1}). The activity of the Qα91A/Wα92A mutant constituted 5.4 μmole*min^{-1}*mg^{-1}.This implies the crucial role of the Dα120. We assume that the decrease of activity of the triple mutant is due to the destabilization of the BrdGLA tetrameric structure, which leads to dissociation of functionally active tetramer to inactive dimers. Additional experiments were conducted to get more insight into oligomeric state of BrdGLA. In particular, we studied the stability of the mutants under neutral conditions at 37°C. Results are presented in Fig. 5. Introduction of the described α-subunit mutations led to the decrease in stability of all mutants as compared to the wild-type enzyme. However, the nature of the stability change was different for different mutants and ambiguously correlated with the data on enzyme activity. For example the activity of the double Qα91A/Wα92A mutant was insignificantly decreased and constituted 71% of the wild type enzyme. At the same time, this mutant showed the worst stability: 80% of activity was lost after 20 h of incubation (Fig. 5, line 4). Interestingly, the rate of inactivation of the double Dα121A/Iα122A mutant (Fig. 5, line 2), that has the highest activity, is slower than that of the triple mutant (Fig. 5, line 3): After 45 hours of incubation the double mutant retains 74% of its original activity, whereas the activity of the triple mutant drops to 52%. Thus, the values of mutant stability correlate well with the values of their activity.

In general, the correlation between mutant stability and activity is rather good and corresponds well to the predicted role of the replaced residues in the formation of the intersubunit contacts. In all cases, the substitution of amino acid residues located in the proposed interface area to alanine results in enzyme destabilization.
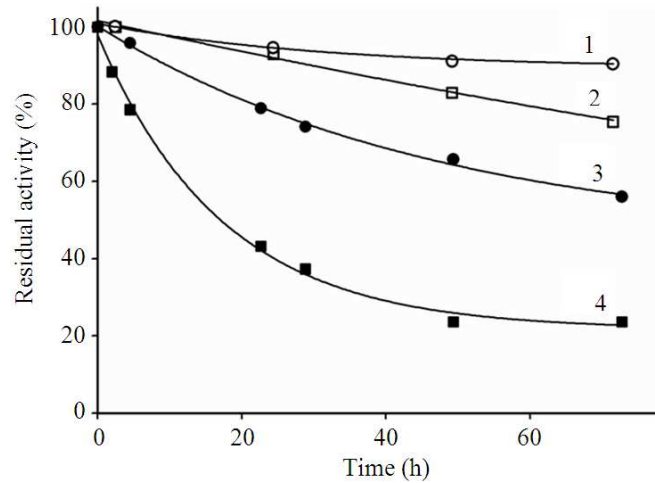
Fig. 3. Stability of the wild type of BrdGLA (lines 1 and 4) and the Kβ198A/Qβ50Nmutant of BrdGLA (lines 2 and 3) at neutral (pH 7.0, lines 1 and 2) and alkaline (pH 10.0, lines 3 and 4) conditions at 35°C
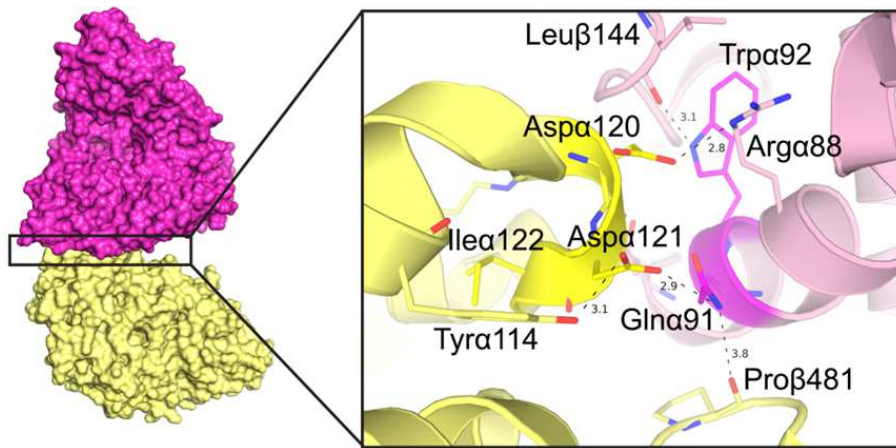

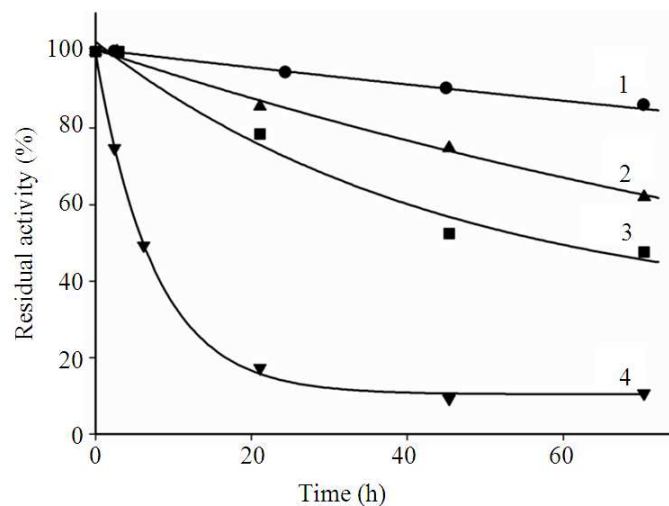Fig. 4. Interface area between the two α/β dimers of the BrdGLAheterotetramer

Fig. 5. Stability of the α-subunit mutants of BrdGLA at neutral conditions (0.1 M KH_2PO_4, pH 8.0, 37°C): 1-the wild type BrdGLA, 2-the Dα120A/Dα121A/Iα122A mutant, 3-the Dα121A/Iα122A mutant, 4- the Qα91A/Wα92A mutant

Most mutants possess lower activity, indicating important role of the tetramer for BrdGLA functionality. Partial increase in the activity in the double Dα121A/Iα122A mutant may be due to the greater availability of the active center. Introduction of the third Dα120A mutation leads to lowering of activity and stability values. This implies that this residue is critical for the maintenance of functional, stable state of the enzyme.

Mutants of Beta-Subunit

The third region of the dimer-dimer interface comprises four consecutive amino acid residues within the β-strand in the C-terminus of the β-subunit. These residues are important for ionic interactions and hydrogen bonds with the part of the other dimer containing several adjacent positively and negatively charged amino acids. To elucidate the role of this site we have obtained a series of BrdGLA/ChBD deletion variants by successive elimination of C-terminal amino acids comprising this region. Five constructs were obtained:

- 1-d749-753-carries a deletion of the five terminal amino acids: FNFLP
- 2-d748-753-carries a deletion of the six terminal amino acids: PFNFLP
- 3-d747-753-carries a deletion of seven terminal amino acids: TPFNFLP
- 4-d746-753-carries a deletion of eight terminal amino acids: RTPFNFLP
- 5-d744-753-carries a deletion of ten terminal amino acids: QERTPFNFLP

Recombinant BrdGLA/ChBD variants were expressed and purified using the standard protocol. Proteins with most significant deletions (more than 6 C-terminal amino acids) could not be isolated. The yield of two successfully purified variants d749-753 and d748-753 comprised 200 µg from 30 ml culture, which is five times less than that of α-subunit mutants. The specific activity of d748-753 and d749-753 mutants decreased to 2.6 and 3.0 µmol*min^{-1}*mg^{-1} respectively, constituting 35 and 40% of the activity of the wild-type enzyme (Table 2). Stability measurements have shown that the C-terminal mutants are generally less stable than the wild-type enzyme, but their stability is higher than that of the α-subunit mutants. After incubation during 45 hours the activity of d748-753 and d749-753 mutants dropped to 78% and 64%, respectively. Thus, the mutant with deeper deletion d748-753 has a lower activity compared to the d749-753 mutant and simultaneously the higher stability. Despite the lack of direct correlation between the activity and stability of the two mutants studied, there is no doubt that the

introduced deletions resulted in significant protein destabilization and inactivation, consistent with the proposed role of the C-terminal region of the β-subunit in tetramer formation.

Modification of BrdGLA/ChBD with Glutaraldehyde

Intermolecular cross-linking of protein subunits with different cross-linking agents is a useful method to investigate the protein oligomeric state. We have used the glutaraldehyde cross linking to ascertain the subunit composition of the BrdGLA/ChBD. As evident from the electrophoregram presented on Fig. 6, a large number of additional bands is present in the sample obtained after cross-linking. Besides major bands corresponding to α- and β-subunits (20 and 50 kDa) a distinct band of 80 kDa, corresponding probably to protein dimer and 115 kDa band, likely corresponding to heterotetramer, can be easily identified. Intense distinct higher molecular weight bands (240 kDa and above) are also present. We assume that the 240 kDa band corresponds to protein octamer. Numerous minor bands likely arise due to arbitrary cross-links between the α-β dimer and α-subunit, α-β dimer and β-subunit etc. In general, experiment with glutaraldehyde has shown the abundance of oligomeric forms generated as a result of cross-linking between protein subunits and separate oligomers. The presence of tetramers and higher molecular weight forms generated as results of intermolecular cross linking may indicate the predominance of tetramers in solution of the native enzyme.
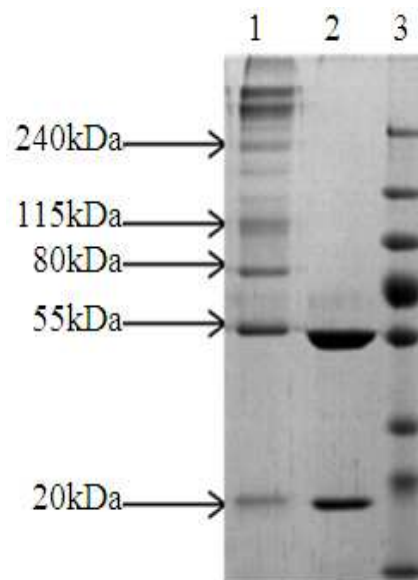

Fig. 6. Separation of crosslinkedBrdGLA/ChBDprotein in a gradient SDS-PAGE

Table 2. Activity and stability of the wild type enzyme and double mutant BrdGLA Kβ198A/Qβ50N at neutral and alkaline pH

| | Specific activity (μmole*min^{-1}*mg^{-1}) | Residualactivity 70 h, 35°C (%) | |
| | | --- | |
Mutant	pH 7.0	pH 7.000	pH 10.0
BrdGLA wild type	7.6	90.000	23.0
BrdGLAKβ198A/Qβ50N	7.2	75.000	56.0

Conclusion

Amino acid residues critical for substrate specificity of *B. diminuta* glutaryl acylase were identified using sequence and structure analysis, molecular modeling and site-directed mutagenesis. The chosen strategy of bioengineering of BrdGLA aimed at changing its substrate specificity have enabled to induce new enzymatic activity and obtain enzyme variants with catalytic activity against cephalosporin C. Through modelling of BrdGLA intersubunit interactions amino acid residues forming contacts between two α-β dimers have been identified. Variants of BrdGLA with replacement of these amino acid residues to alanine are characterized by reduced stability and diminished activity, proving the role of these residues in formation of the contact area between two α-β dimers. Protein cross-linking experiments have shown that various oligomeric forms coexist in the solution with predominance of tetramers. Finally, the double Qβ50N/Kβ198A BrdGLA mutant was obtained that is promising for development of more efficient biocatalysts stable in alkaline medium.

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Author's Contributions

Michael Eldarov: Conceived of the research, designed the study, drafted the paper.

Anna Lyashenko: Performed mutagenesis, protein expression and purification.

Tatyana Sherbakova: Performed activity measurements, protein cross-linking, helped with manuscript preparation.

Dmitry Suplatov and Kirill Kopylov:

Were responsible for computer modeling, in silico design of mutations, finalized the paper.

Vytas Svedas: Was general supervisor and revised the article.

Ethics

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. Each author confirms the manuscript represents honest work. All authors have approved the manuscript. Each author agrees with the order in which his name appears on the title page.

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