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Molecular Modeling of Penicillin Acylase Binding with a Penicillin Nucleus by High Performance Computing: Can Enzyme or its Mutants Possess β-lactamase Activity?

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High-performance computing has been used for molecular modeling of penicillin acylase interaction with a penicillin nucleus 6-aminopenicillanic acid (6-APA) to assess whether the wild-type enzyme or its mutants could possess β-lactamase activity. Applying parallel hybrid GPU/CPU computing technologies for metadynamics calculations with the PLUMED library in conjunction with AMBER software suite it has been shown that trace amounts of wild-type penicillin acylase-6-APA complexes leading to a β-lactamase reaction can be formed. Higher β-lactamase activity can be observed in enzyme mutants by introducing charged residue in the substrate binding pocket and its proper positioning with respect to a catalytic nucleophile, including stabilization of the tetrahedral intermediate in the oxyanion hole. Thus, it has been shown that the certain mutations facilitate the orientation of the substrate required for the manifestation of β-lactamase activity in the penicillin acylase active center.

Keywords: moonlighting protein, penicillin acylase engineering, β-lactam antibiotics resistance, β-lactamase design, metadynamics.

Introduction

In the recent years, we are witnessing inreasing evidences that a "one protein-one function" paradighm is no longer valid and seems to be rather an exception to the rule. Well documented examples of an ability of the same active center to catalyze different chemical conversions as well as a presence in a protein/enzyme molecule of several active sites responsible for various functional properties or catalytic activities have opened new intriguing period in enzymology. The terms of enzyme promiscuity and protein moonlighting are being filled with new experimental data and theoretical substantiation. This recently observed phenomenon is of not only fundamental, but also of significant practical interest, since the presence of novel activities, in addition to the traditionally known and the attributed canonical one, significantly expands our understanding of the role of enzymes in the functioning of living systems, as well as options for their applications [7, 10, 13]. In this context, it is interesting to take a look at the enzymatic transformations of β-lactam antibiotics.

Two types of enzymes that convert the same substrate penicillin are the most known: penicillin acylases capable of hydrolyzing the more stable amide bond formed by the side chain acyl group with the antibiotic nucleus [20] and β-lactamases which cleave the less stable amide bond of the β-lactam ring in the antibiotic nucleus, thus irreversibly inactivating it [15]. Despite the canonical catalytic activities of these two enzyme families are quite different, there are some experimental facts to suppose that penicillin acylases may have minor β-lactamase activity and thus possess moonlighting properties. It is based on the experimental observation that at the penicillin acylase-catalyzed industrial conversion of penicillin to produce a penicillin nucleus 6-aminopenicillanic acid there is some accumulation of the β-lactam hydrolysis products along

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with a formation of the target product. In this regard, the question of whether a side reaction of hydrolysis of the β-lactam ring can occur in the active center of penicillin acylase and whether mutations in the enzyme structure can increase such a minor nonspecific activity is of interest. The answer to this question may also help to understand whether, in the course of evolution, with random mutations, penicillin acylase can become a resistance factor to the action of β-lactam antibiotics. Molecular modeling can be used for evaluation of potential moonlighting activity of an enzyme, especially when this activity is much lower compared to the canonical one and therefore is rather complicated to characterize it experimentally what is expexted with the β-lactamase activity of penicillin acylases.

The ability of an enzyme to convert one or another substrate is primarily determined by its recognition and binding in the active center of the enzyme followed by a proper orientation toward the catalytic amino acid residues and conversion to the products. The search over the entire phase space which describes all possible conformational and orientational states of the substrate in the active site is a resource-intensive task, therefore usually specialized methods are used which are limited to either the search and evaluation of the final most favorable bound state (molecular docking [6], MMPBSA calculation [11], thermodynamic integration [22], free energy perturbation [26] methods) or partial analysis of the substrate delivery paths (implementations of steered molecular dynamics [8] and umbrella sampling [12]). One of the advanced implementations of the search for the substrate phase space is funnel metadynamics [14] which allows not only to analyze substrate binding in active centers of different configuration but also explore alternative bindings as well as physically accessible routes of substrate delivery with subsequent estimation of the binding energies for these states. The method is based on the advanced sampling technology – metadynamics implemented in the PLUMED software package which works with a variety of molecular modeling programs [2, 23]. To study the possible major and alternative prereaction states of the penicillin acylase and β-lactamase substrate (6-APA), it is important to take into account not only the configuration of the active site, but also the orientational possibilities of the substrate itself and therefore the redefinition of the so-called collective variables – generalized coordinates of ligand binding in the active site – is needed. Possibilities of recent incorporation of metadynamics to high performance molecular dynamics package AMBER [4] utilizing GPU allow efficient sampling of states without the need to limit the diffusion of the substrate in the solvent when approaching the active site [5]. The protocol used in this article makes it possible, using parallel hybrid GPU/CPU computing technologies, to calculate both a significant amount of the phase space of the enzyme-substrate interaction for both the wild-type enzyme and selected mutant forms with the expected activity.

1. Material and Methods

The classical approach to assessing the energy of the chemical stages of enzymatic transformations is the choice of a reaction coordinate and QM/MM simulation to obtain an energy profile, however these calculations are time as well as computational resource consuming and it is practically impossible to perform such studies for a wide range of enzyme mutants and their potential substrates. As a practical alternative the criteria of theoretical chemistry based on the analysis of the so-called reactive conformational states ("Near-to-Attack Conformations", NAC) can be used to estimate the energy landscape of enzymatic reactions which began to be actively used along with the methods of molecular dynamics [3, 19]. The use of this approach makes it possible not to perform sophisticated QM/MM calculation but to focus on the analysis

of the classical molecular dynamics trajectory in the characterization of native enzymes and the selection of their mutant forms with new properties.

In metadynamics, the free energy profile is reconstructed by introducing an additional bias potential, which makes it easier for the system to overcome potential barriers and move to other regions of the phase space [23]. The bias potential acts through selected collective variables which are functions of the atomic coordinates. Accordingly, NAC-criteria might serve as prospective collective variables. To assess the possibility of enzymatic hydrolysis of amides in metadynamics (to classify each particular conformation as capable or not capable of entering into a subsequent chemical transformation) it is possible to propose two criteria (Fig. 1) – the angle of the nucleophilic attack on the plane of the hydrolysable amide bond (β) and the distance between a nucleophile atom and an accepting amide carbon atom (d). A conformation is considered reactive if the angle β lies in the range of 70–110° and the distance d is less than or equal to the sum of the van der Waals radii of the nucleophile and electrophile. In the case of hydrolysis of 6-APA by penicillin acylase the nucleophile is the oxygen atom of the hydroxyl group of the bS1 residue, the electrophile is the carbon atom of the amide bond in the β-lactam group and the threshold value for d is 3.5Å. It is justified to use the angle of rotation of the substrate relative to the axis (the line connecting bW154 and bY376 residues, the residues are labeled with small letter depicting the chain, if available, followed by one-letter code of original amino acid residue and its position; one-letter code of new residue is added to the end if mutation is considered) passing through the center of the penicillin acylase hydrophobic pocket as the third collective variable ϕ (the orientation of the substrate with the amide part directed into the hydrophobic pocket corresponds to β=0°) which in addition to the positional search with respect to the variable d will expand the search for various substrate orientations.

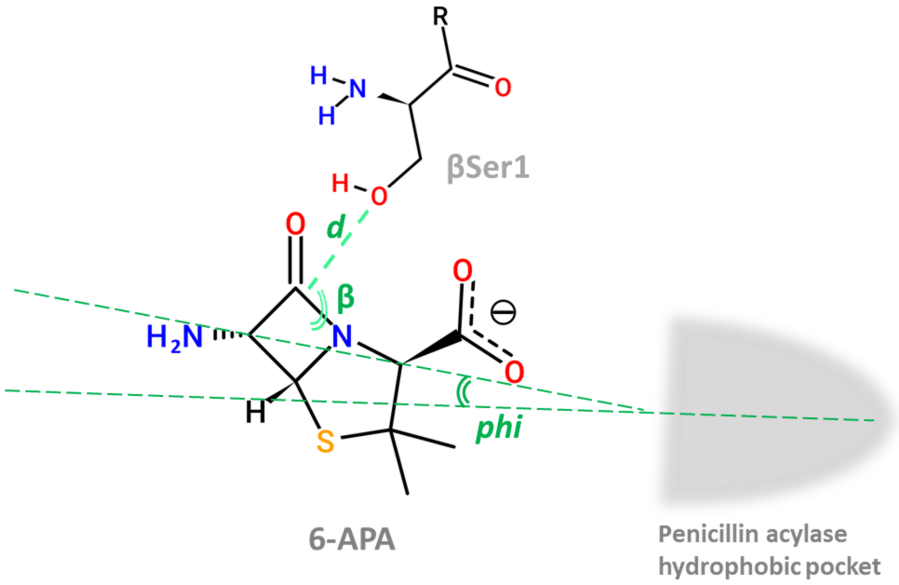


Figure 1. Collective variables (d;β;ϕ) used to perform metadynamics in the penicillin acylase-6-APA complex. d corresponds to the distance between a nucleophile atom and an accepting amide carbon atom, β corresponds to the angle of the nucleophilic attack on the plane of the hydrolysable amide bond and ϕ(phi) is the angle of rotation of the substrate relative to the axis passing through the center of the penicillin acylase hydrophobic pocket

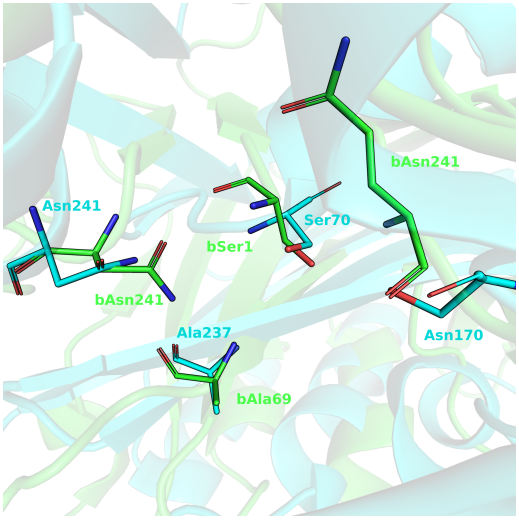
To calculate the energy landscape of various enzyme-substrate complexes and determine the reaction states the metadynamics has been performed using the phase space of three collective variables (d;β;ϕ) while imposing the wall potential to restrict nucleophilic attack distance d and prevent the substrate to leave active site area ($>$ 10Å).

For molecular dynamics calculations the AMBER18 package was used [4]; for metadynamics calculations – the open-source, community-developed PLUMED library [2] version 2.6 [23] in conjunction with AMBER18. Recently developed PLUMED incorporation to AMBERs pmemd program to resolve performance issues within hybrid molecular dynamics/metadynamics runs utilizing GPU units has helped to gain free energy landscape convergence within valuable timing period [5]. As a result, the computational performance of the AMBER18+PLUMED bundle performed approximately 4 times faster than the NAMD 2.13 [17]+PLUMED bundle under similar conditions (the systems under study of the order of 80,000 atoms and more). The enzyme-6-APA systems (native or mutant forms of the enzyme) were preliminarily prepared by removing low molecular substances from the initial structure (RCSB 1gm9); protons were added to the protein according to pH 7.5 using the pdb2pqr30 utility [9]; geometry of 6-APK was optimized in package GAMESS [21] using hybrid density functional B3LYP with the 6-31+G* basis set followed by deriving the point atomic charges using RESP method at the RED server [24]. A system was created for enzyme-substrate complexes that included the TIP3P solvent in which the distance from the edge of periodic box to any atom of solute was no less than 15Å. Cl– and Na+ ions (up to 0.1M concentration) were added to the system to create conditions close to physiological ones. The total charge of the system was adjusted to zero by adding corresponding ions. Energy minimization was carried out for each system (2500 steps) followed by heating to 300 K for 60 ps, lifting of limitations from heavy atoms for 290 ps and relaxation for 5 ns in the NPT ensemble with a gradual adjustment of the density to the constant value of 1.04 g/cm^3. Standard simulation in solution was performed with the AMBER18 package at 300 K with the integration step of 2 fs under conditions of the NVT ensemble. The system temperature was controlled using a Langevin thermostat [16]. The height of the Gaussian hills in metadynamics was set to the initial value of 0.6 kcal/mol; the width was set 0.01, 0.0075 and 0.1 1 for d, β and ϕ, respectively. The well-tempered variant of metadynamics was used by setting BIASFACTOR value to 13 [1]. New potentials were added every 300 steps at the integration step of 2 fs. The temperature of simulation was set to 300 K. Plumed library restraining potential UPPER_WALLS was applied to distance collective variable at 10Å with KAPPA value 2500 to limit the phase space accessible during simulation. Ten parallel calculations with shared metadynamics potential (multiple-walkers technique) were used to accelerate calculations [18]. The convergence of metadynamics was estimated from the time dependence of the difference in free energy values at each minima point of (d;β;ϕ) energy landscape.

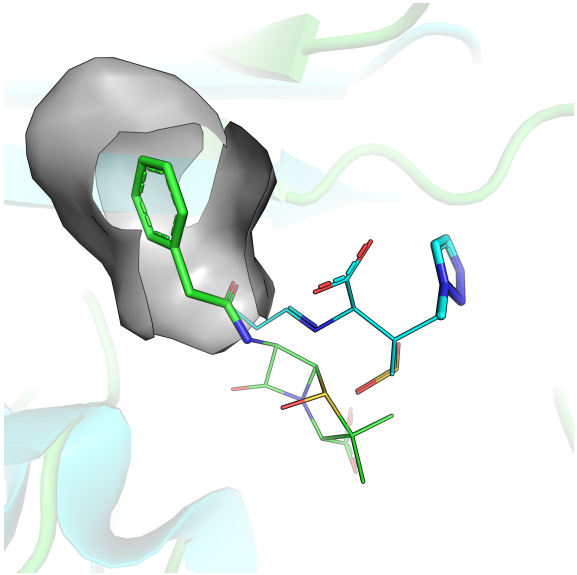
2. Results and Discussion

When considering the catalytic mechanisms of penicillin acylase and β-lactamase it should be noted, first, that the nucleophile of penicillin acylase is formed by N-terminal serine residue while β-lactamase uses a non-terminal serine residue as a nucleophilic reagent. Pairwise structural superposition of enzymes by key catalytic residues including serine residues as well as residues involved in the stabilization of the oxyanion bAla69 and Ala237, bAsn241 and Asn170, bGln23 and Ser130 of penicillin acylase and β-lactamase, respectively, demonstrates spatial matching of the catalytic cores but a low similarity index for the entire protein structure (Fig. 2a). The

enzymes do not share common protein fold and belong to different superfamilies while sharing analogous substrate amide group recognition. Enzyme substrates from superimposed crystallographic structures are rotated relative to each other by 180 degrees: the acyl part of the substrate is located in the hydrophobic pocket of penicillin acylase contrary to bulk water orientation in β-lactamase (Fig. 2b).



(a) bSer1-Ser70, bAla69-Ala237, bAsn241-Asn170, bGln23-Ser130 pairs of residues of penicillin acylase (1gm9, green sticks) and β-lactamase (4a5r, cyan sticks), respectively, were used for superposition (RMSD = 1.2Å)



(b) Substrates orientation in the superimposed crystallographic structures of pencillin acylase (1gm9, green) and β-lactamase (4a5r, cyan). The acyl groups of the substrates are shown as bold sticks; the surface represents penicillin acylase hydrophobic pocket location

Figure 2. Pairwise structural superposition of penicillin acylase and β-lactamase by active site amino acid residues

The study of the orientational and conformational states of 6-APA in the active site of penicillin acylase have been carried out by means of metadynamics in the selected collective variables (d;β;ϕ) followed by reconstruction of the free energy landscape. The review of the landscape obtained for wild-type penicillin acylase reveals lack of stabilized regions corresponding to the required orientation of 6-APA to support β-lactamase activity (Fig. 3). For the hydrolysis of the β-lactam ring to proceed, it is necessary the attack angle β be around 90° while the nucleophilic attack distance d be less than 3.5Å: in the case of wild-type penicillin acylase low-energy states conferring such parameters are absent. The shortest distance from catalytic bSer1 to amide bond carbon d corresponded to 2 regions – region 1 and region 2 in the Fig. 3. In region 1, the distance d varies from 3.5 to 5Å, the attack angle β do not exceed 25°. In region 2, the distance varies from 2.5 to 3.5Å but attack angle β do not exceed 50° turning the substrates amide group to the bulk water. This orientation is supported by the formation of a hydrogen bond between the carboxyl group of 6-APA and the OH group of bSer386 however no states are observed in which the stabilization of the transition state in the oxyanion hole is possible. Even though it is possible to isolate individual frames of the molecular dynamics trajectory that corresponds to the correct orientation of the substrate ($d < 3.5$Å, $\beta \approx 90°$) as well as formation of hydrogen bonds between 6-APA carbonyl oxygen and the bAla69 main chain hydrogen and bAsn241 side

chain hydrogen it can only explain the trace activity of the enzyme in purified preparations (purification by affinity chromatography, unpublished data).

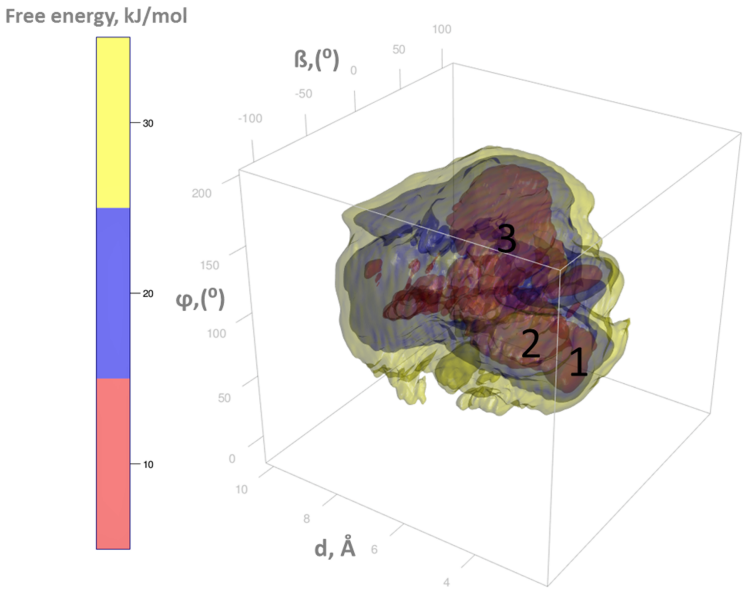


Figure 3. Free Energy Landscape of the 6-APA/(wild type)penicillin acylase binding is reconstructed using well-tempered metadynamics as a function of nucleophilic attack distance d, the attack angle β and the angle of rotation of the substrate ϕ. The landscape is rendered using isometric surfaces with a step of 10 kJ/mol. Areas corresponding to major minima are labeled by numbers

Mutant forms of penicillin acylase capable of efficiently catalyzing alternative reactions could evolve in the course of natural selection in a variety of ways. Thus for β-lactamase activity it would be reasonable to introduce a new nucleophilic serine residue next to the amide bond of the β-lactam ring, however, then it would be necessary also to move the oxyanion hole due to additional mutations. Such an interference could affect the maturation of the enzyme. The shortest one for β-lactamase reaction seems to be introduction of the charged residue into the substrate binding pocket thus moving the β-lactam amide bond toward bSer1 nucleophilic attack. Given the trace presence of the corresponding orientations of 6-APA in the active site of the wild-type penicillin acylase an evolutional path leading to further stabilization of these states would be an option. In order to identify corresponding penicillin acylase mutants with β-lactamase activity, it is necessary to select substitutions that allow to bind and orient the 6-APA in the enzymes active site properly directing the substrate carboxyl group with respect to the catalytic nucleophile residue, and then, if necessary, select additional mutations. In order to check adequacy of this hypothesis we have modelled the introduction of a positively charged amino acid residue within the substrate binding pocket.

With the introduction of bV56R substitution stabilized states appear on the free energy landscape which correspond to the substrate orientation required for β-lactam hydrolysis to occur (Fig. 4, the region 1).

The nucleophilic attack distance d in these states reaches the limit of 2.75Å, the attack angle β is 60–90°, the substrate is oriented in the required manner relative to the hydrophobic pocket (ϕ = 40–50°).

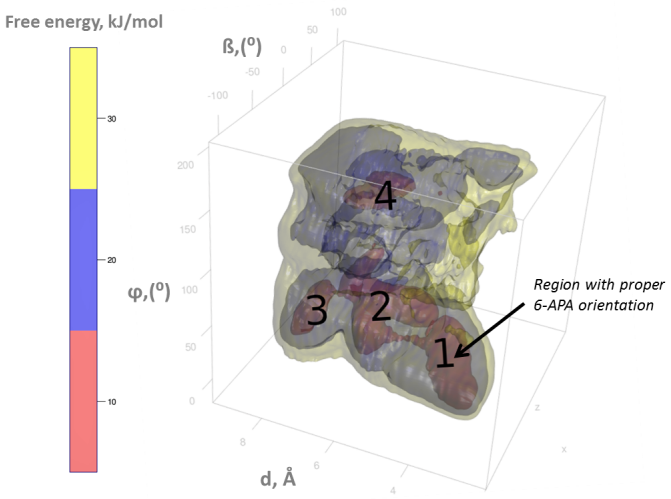


Figure 4. Free Energy Landscape of the 6-APA/(bV56R)penicillin acylase binding is reconstructed using well-tempered metadynamics as a function of nucleophilic attack distance d, the attack angle β and the angle of rotation of the substrate ϕ. The landscape is rendered using isometric surfaces with a step of 10 kJ/mol. Areas corresponding to major minima are labeled by numbers

We also proposed to additionally introduce the substitution of bA69 for a less bulky glycine residue (bA69G) so that the substrate molecule could more effectively approach the catalytic bSer1 from solution. The double mutant bV56R+bA69G retains a region with the 6-APA orientation required for the reaction to proceed (indicated by the arrow in Fig. 5) in which the substrate is oriented with the carboxyl group into the hydrophobic pocket: the collective variables vary within the appropriate limits (d = 2.75–3.5Å, β = 60–90, ϕ = 40–60). For double

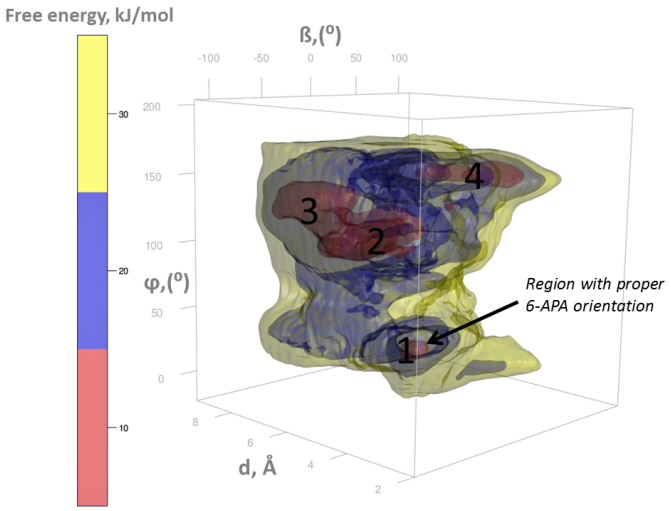


Figure 5. Free Energy Landscape of the 6-APA/(bV56R+bA69G)Penicillin Acylase binding is reconstructed using well-tempered metadynamics as a function of nucleophilic attack distance d, the attack angle β and the angle of rotation of the substrate ϕ. The landscape is rendered using isometric surfaces with a step of 10 kJ/mol. Areas corresponding to major minima are labeled by numbers

penicillin acylase mutant 6-APA orientation completely fulfill stabilization of amide bond within oxyanion hole directing the carbonyl oxygen towards bAla69 and bAsn241 residues (Fig. 6).

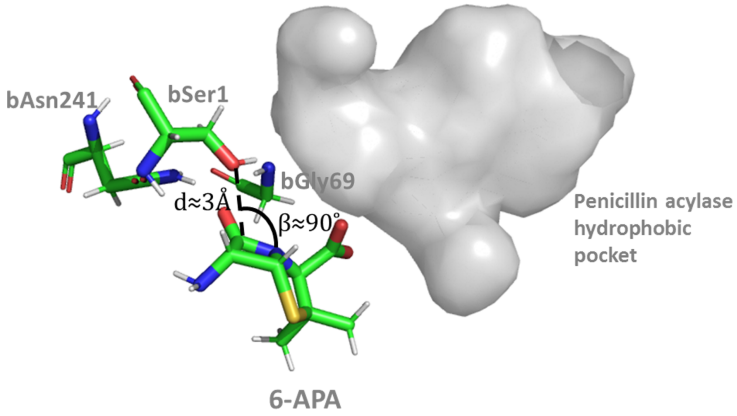


Figure 6. Orientation of 6-APA in the active site of the penicillin acylase double mutant bA69G+bV56R which completely fulfills β-lactam hydrolysis transition state stabilization by oxyanion hole formed by bAla69 and bAsn241 residues

When analyzing the states corresponding to different energy minima on the free energy landscape, a large number of conformations were revealed, in which the carboxyl group of 6-APA forms a hydrogen bond with the aR145 residue. In order to reduce the proportion of such states in favor of the more preferable orientation for the alternative β-lactamase reaction (when the carboxyl group is directed to the bV56 residue), it is also reasonable to propose aR145G substitution. For the bA69G+bV56R+aR145G mutant the free energy landscape has also been constructed to support the major minima of β-lactamase-like substrate orientation (Fig. 7, indicated by the arrow).

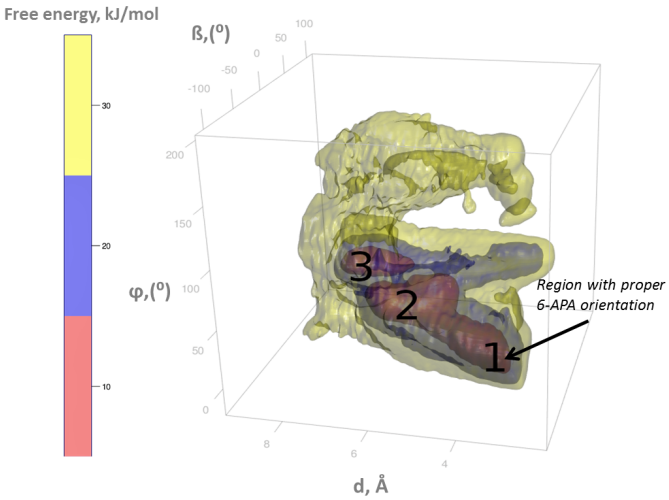


Figure 7. Free Energy Landscape of the 6-APA/(bV56R+bA69G+aR145G)penicillin acylase binding is reconstructed using well-tempered metadynamics as a function of nucleophilic attack distance d, the attack angle β and the angle of rotation of the substrate ϕ. The landscape is rendered using isometric surfaces with a step of 10 kJ/mol. Areas corresponding to major minima are labeled by numbers

Conclusion

High-performance computing has been used for molecular modeling of penicillin acylase interaction with a penicillin nucleus 6-aminopenicillanic acid (6-APA) to assess whether the wild-type enzyme or its mutants could possess β-lactamase activity. Despite the trace amounts of wild-type penicillin acylase-6-APA complexes leading to a β-lactamase reaction have been shown to be formed, the manifestation of β-lactamase activity by a wild-type penicillin acylase is unlikely. However β-lactamase activity can be observed due to penicillin acylase mutations when charged residue is introduced in the substrate binding pocket leading to its proper positioning with respect to a bSer1 nucleophilic attack, including stabilization of the tetrahedral intermediate in the oxyanion hole. It has been shown that the mutations bV56R, bA69G and aR145G can facilitate the orientation of the substrate required for the manifestation of β-lactamase activity in the penicillin acylase active center.

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