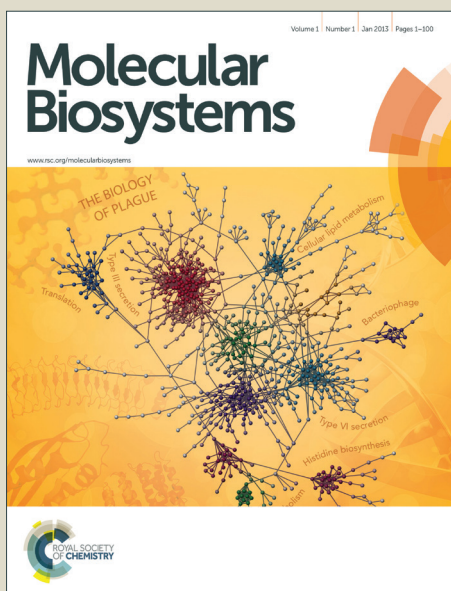


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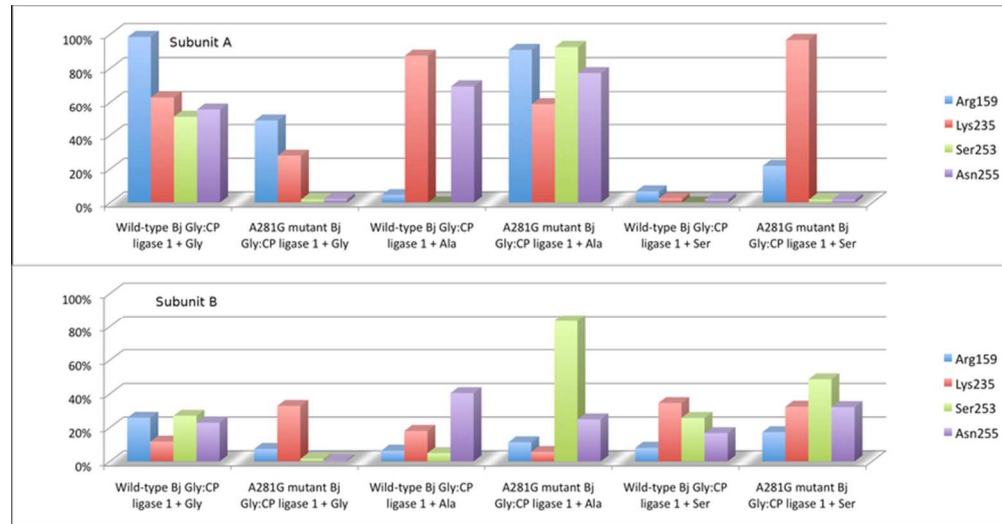
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## ARTICLE

## A single amino acid substitution affects the substrate specificity of the seryl-tRNA synthetase homologue

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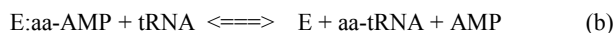
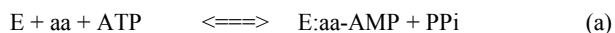
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Recently described and characterized *Bradyrhizobium japonicum* glycine:[carrier protein] ligase 1 (Bj Gly:CP ligase 1), a homologue of methanogenic type seryl-tRNA synthetase (SerRS) is an intriguing enzyme whose physiological role is not yet known. While aminoacyl-tRNA synthetases supply ribosome with amino acids for protein biosynthesis, this homologue transfers the activated amino acid to a specific carrier protein. Despite remarkable structural similarity between the Bj Gly:CP ligase 1 and the catalytic core domain of methanogenic type SerRS, the ligase displays altered and relaxed substrate specificity. In contrast to methanogenic SerRS which exclusively activates serine, the Bj Gly:CP ligase 1 predominantly activates glycine. Besides, it shows low activity in the presence of alanine, but it is incapable of activating serine. The detailed computational study aiming to address this unexpected substrate specificity toward the small aliphatic amino acids revealed the A281G Bj Gly:CP ligase 1 mutant as the most promising candidate with reconstituted catalytic activity toward the larger substrates. The A281G mutation is predicted to increase the active site volume, allowing alanine and serine to establish important hydrogen bonds within the active site, and to adopt an optimal orientation for the reaction. The results were tested by the site-directed mutagenesis experiments coupled with *in vitro* kinetic assays. It was found that the A281G substitution greatly affects the enzyme specificity and allows efficient activation of both polar and small aliphatic amino acids (serine, glycine and alanine), confirming predictions and conclusions based on molecular dynamics simulations.

### Introduction

The aminoacyl-tRNA synthetases (aaRSs) belong to a group of enzymes that catalyse the attachment of specific amino acids to their cognate tRNA molecules in a two-step reaction.<sup>1</sup> The initial step results in the formation of a stable enzyme-bound aminoacyl-adenylate intermediate (a). In the second step, the amino acid moiety of the enzyme-bound intermediate is transferred to the 3'-end of the cognate tRNA(s) (b).



According to their structural diversity, aaRSs are divided into two classes.<sup>2</sup> Seryl-tRNA synthetases (SerRSs) belonging to class II enzymes are phylogenetically of particular interest since they have developed two different modes of amino acid binding. There are a standard (bacterial) SerRSs, found in the majority of organisms and atypical (methanogenic) SerRSs, found only in methanogenic archaea. Methanogenic SerRSs employ zinc-mediated serine recognition,<sup>3</sup> while bacterial SerRSs use exclusively active site amino acid moieties for serine binding. The SerRS-like proteins further enrich the diversity of the SerRSs family. These SerRS-like proteins, although structurally related, poses many different, non-

canonical functions. Some of them are involved in antibiotic production<sup>4,5</sup> and resistance.<sup>6</sup> For the SerRS-like protein found in insects important, but undefined function in respiration and mitochondrial physiology<sup>7</sup> has been proposed.

The main focus of the present research has been the recently described and characterized SerRS-like proteins<sup>8</sup> that show remarkable structural similarity to the catalytic core domain of methanogenic-type SerRS, despite low sequence identity between these enzymes. They possess signature motifs of class II aaRSs (motifs 1, 2, 3) and two idiosyncratic features, the loop-helix (LH) element and helix-turn-helix (HTH) motif (Fig. S1, ESI). These homologs activate amino acids, but lack canonical tRNA aminoacylation activity since they are deprived of the tRNA-binding domain. These activated amino acids are then transferred onto the specific carrier proteins (CPs) posttranslationally modified with a 4-phosphopantetheine.<sup>8</sup> Considering the abovementioned reaction, these enzymes are named as amino acid:[carrier protein] ligases. Two *Bradyrhizobium japonicum* enzymes preferentially activate glycine, but also show enzyme activity in the presence of alanine. Surprisingly, these enzymes do not show serine activation at all, although they are homologs of methanogenic SerRSs. On the other hand, the *Agrobacterium tumefaciens* homologue is more relaxed in the amino acid recognition; it

prefers alanine over glycine and it is capable of activating serine as well.<sup>8</sup> Amino acid recognition other than serine is puzzling since the amino acid specificity of aminoacyl-tRNA synthetases is evolutionary strictly conserved. It has been proposed that the changed amino acid specificity toward the small aliphatic amino acids, Gly and Ala could be explained by the lack of the transition state stabilizing interactions.<sup>8</sup> Namely, Arg353 that helps in the orientation of hydroxyl-group of serine in the active site of atypical SerRS form *Methanosarcina barkeri* (Mb aSerRS), is replaced by Met174 in *B. japonicum* glycine:[carrier protein] ligase 1 (Bj Gly:CP ligase 1). The same Arg to Met substitution can be found in *A. tumefaciens* aa:CP ligase, but the *A. tumefaciens* enzyme more efficiently activates alanine than glycine. Since the *A. tumefaciens* enzyme shows activity in the presence of serine as well, the observed Arg to Met substitution does not fully explain the altered aa:CP ligase amino acid selectivity. Further examination of the Bj Gly:CP ligase 1 and the aMbSerRS active sites, coupled with inspection of the sequence alignment, pinpointed Gly296 in *A. tumefaciens* aa:CP ligase, corresponding to Gly463 and Ala281 in Mb aSerRS and Bj Gly:CP ligase 1, respectively, as a possible cause of the observed differences in substrate specificity between the enzymes. In order to elucidate the determinants of the substrate specificity in *B. japonicum* Gly:CP-ligase 1, we have focused on the first step of enzyme reaction that involves the amino acid attack at the  $\alpha$ -phosphate of ATP resulting in the formation of the aminoacyl-adenylate and the inorganic pyrophosphate (PPi). To address this altered substrate specificity we performed molecular dynamics (MD) simulations assisted by *in vitro* site-directed mutagenesis and kinetic analysis of wild-type and mutant enzymes. MD simulations have shown, and experimental kinetic analyses have confirmed, that different amino acid specificity of the *B. japonicum* enzyme can be obtained by a single active-site mutation, efficiently converting the specificity of *B. japonicum* enzyme to the specificity inherent *A. tumefaciens* counterpart.

## Materials and Methods

### Model setup

The initial models for molecular dynamics (MD) simulations were built using two out of several Bj Gly:CP ligase 1 structures available in the Protein Data Bank (PDB). In one of them the enzyme is complexed with AMP (PDB code 3MF2, resolution 2.15 Å) and in the other it is complexed with glycyl-adenylate analogue (PDB code 3MF1, resolution 2.20 Å). In both structures the protein exists as a homodimer with one zinc cation in each subunit. The missing residues in the chain were modelled using the program Modeller.<sup>9</sup> Hydrogens were added to the protein atoms using the tleap module of the AMBER12 suite.<sup>10</sup> All Arg and Lys residues were positively charged and all Glu and Asp residues were negatively charged, as expected under the physiological conditions. Protonation states of histidine residues were determined according to their ability to

form hydrogen bonds with neighbouring amino acid residues. We studied the ligand-free enzyme and its complexes with ATP and the selected amino acid ligand. Charges on the active site Zn cation and on the Mg cation that bridges  $\alpha$ - and  $\beta$ -phosphate of ATP were set to 1.5 e<sup>+</sup>, according to the quantum mechanical (QM) calculations on model systems (see below). The other two Mg cations were treated as divalent ions (Mg<sup>2+</sup>), amino acid ligand was treated as monoanion, [amino acid]<sup>1-</sup>, while ATP was fully deprotonated (ATP<sup>4-</sup>). VMD plugin Molefacture was used to build substrates, glycine, serine and alanine from the glycyl-adenylate analogue. The experimentally determined structures with PDB codes 3MF2 and 3MF1 were overlapped in order to tune the mutual position of the ligands in the protein active site. The superimposed catalytic sites of different class II enzymes revealed the strictly conserved position and conformation of the aminoacyl-adenylate. Moreover, for each of the substrates, ATP and amino acid, it has been shown to bind according to its corresponding counterparts in the aminoacyl-adenylate complex.<sup>11</sup> Therefore, the position of the adenylate-analog served as a reference for initial positioning of the amino acid and ATP into the enzyme active site. Furthermore, initial positions of the substrates were validated through the structure overlapping between the constructed models and the PDB structures coded as 3MF1 (Bj Gly:CP ligase 1 complexed with glycyl-adenylate), 2CJ9 and 2CJB (*M. barkeri* seryl-tRNA synthetase complexed with seryl-adenylate and serine, respectively). Experimental studies have confirmed that the magnesium cations are required for proper positioning of ATP into the enzyme active site. However, the optimal number of Mg<sup>2+</sup> ions in the active site and their positions are not known. Since the Mg<sup>2+</sup> ions are not present in the experimentally determined structures (3MF1 and 3MF2) used in our study, we examined about 30 PDB structures of class II aaRS and overlapped them with our constructed protein models in order to define the correct positions of metal ions.

Ligands used in the simulations were prepared using antechamber, the module available as part of AMBERTools12. The parameterization was performed within the GAFF force field<sup>12</sup> using the bcc method<sup>13,14</sup> for charge determination. The proteins were parametrized within the ff12SB force field.<sup>15</sup> Structures were solvated in an octahedron box of TIP3P of water molecules ensuring a 10 Å thick buffer of solvent around the protein.<sup>16</sup> To each complex 3 Na<sup>+</sup> were added for neutralization. The resulting systems consisted of ~48 000 atoms (~13 000 molecules of water).

An inspection of the active site residues has revealed amino acid candidates for the selectivity switch. Guided by the active site analysis and differences in the primary sequences of the proteins (Bj Gly:CP ligase 1, *A. tumefaciens* alanine:[carrier protein] ligase (At Ala:CP ligase) and *M. barkeri* SerRS) we built the A281G mutant 3D structure of Bj Gly:CP ligase 1 using SDM predicting web server for site-directed mutants construction.<sup>17</sup> Our aim was to examine how this single mutation affects the active site dynamics and the substrate accommodation into the active site.

Altogether, we built nine molecular models (see Table 1).

**Table 1** Molecular models used in molecular dynamics simulations.

Model number	Molecular model
1	Bj Gly:CP ligase 1 complexed with ATP, Mg ions and glycine
2	Bj Gly:CP ligase 1 complexed with ATP, Mg ions and alanine
3	Bj Gly:CP ligase 1 complexed with ATP, Mg ions and serine
4	Bj Gly:CP ligase 1 (A281G) complexed with ATP, Mg ions and serine
5	Bj Gly:CP ligase 1 (A281G) complexed with ATP, Mg ions and alanine
6	Bj Gly:CP ligase 1 (A281G) complexed with ATP, Mg ions and glycine
7	Bj Gly:CP ligase 1 monomer complexed with ATP, Mg ions and glycine
8	Ligand-free Bj Gly:CP ligase 1 dimer
9	Ligand-free Bj Gly:CP ligase 1 monomer

The performed simulations can be categorized into two main groups. The first group of simulations, aimed to clarify altered substrate specificity of Bj CP:Gly ligase 1, comprises six simulated dimeric protein structures complexed with both substrates, amino acid and the ATP-Mg complex. Structures 1-3 correspond to wild type Bj Gly:CP ligase 1 complexed with ATP, Mg cations and either glycine, alanine or serine, and structures 4-6 correspond to mutant (A281G) Bj Gly:CP ligase 1 complexed with ATP, Mg cations and either glycine, alanine or serine. The second group of simulations, performed on the last three systems from the Table 1, was aimed to examine a) the influence of substrate binding to the enzyme active site and b) the role of structural motifs in the formation of the biologically relevant homodimeric enzyme.

#### Charge correction for metal ions and ligands

The partial charges for Zn and Mg cations were determined by the QM calculations on the model systems. The first model consisted of Zn cation and the ligands from its first coordination shell, namely, the amino acid substrate and the side chains (starting from C $\beta$  atom) of Cys131, Cys279 and Glu176. The second model, used to calculate the Mg charge, consisted of three Mg cations, methyl-triphosphate, representing an ATP molecule, acetic acid, representing invariant Glu237 and the amino acid substrate. We calculated electrostatic potential (ESP) derived atomic charges for Zn cation using HF/6-311G(d,p) method while the Mg charges were determined using DFT (B3LYP/6-31G(d,p)) approach. Calculations were performed by Gaussian 09.<sup>18</sup> Based on the QM calculations, the effective charges of Zn cation and Mg cation close to the  $\alpha$ -phosphate of ATP, were rounded to +1.5|e|.

#### Molecular Dynamics Simulations

The MD simulations were carried out with the AMBER12 suite<sup>10</sup> using the constant pressure periodic boundary conditions. Prior to MD simulations, the protein geometry was optimized in four cycles with different constraints. In the first

cycle (1500 steps), water molecules and the substrate were relaxed, while the protein and zinc cation were constrained using a harmonic potential with a force constant of 32 kcal/(mol $\text{\AA}^2$ ). In the second (2500 steps), third (1500 steps) and fourth cycle (5000 steps), the same force constant of 32 kcal/(mol $\text{\AA}^2$ ) was applied to the zinc cation, while the protein backbone was constrained with only 10 kcal/(mol $\text{\AA}^2$ ). The energy minimization procedure, consisting of 470 steps of steepest descent followed by the conjugate gradient algorithm for the remaining optimization steps, was the same in all cycles. Optimized systems were heated from 0 to 300 K during 30 ps using NVT conditions. This is followed by the 41.03 ns of MD simulations at constant temperature (300 K) and constant pressure (1 atm), with the first 30 ps considered as equilibration. The temperature was held constant using Langevin thermostat with a collision frequency of 1 ps<sup>-1</sup>. For the first 6 ns of productive run a time step of 1 fs was used and for the rest of MD simulations the time step was 2 fs. Bonds involving hydrogen atoms were constrained using the SHAKE algorithm.<sup>19</sup> Structures were sampled every 1 ps over the first 6 ns and every 0.5 ps for the remaining period. The Particle Mesh Ewald method<sup>20</sup> was applied to calculate long-range electrostatic interactions. The nonbonded interactions were truncated at 10.0  $\text{\AA}$ .

#### Data analysis

Structure (RMSD, hydrogen bonds, distances) and dynamics (RMSF, B factors) of the enzyme and ligands were analysed using the module 'ptraj' available in the AmberTools12 and VMD.<sup>21</sup> An interaction was treated as a hydrogen bond if the donor-acceptor distance was  $\leq 3.0 \text{\AA}$  and the donor-hydrogen-acceptor angle was within the range  $180^\circ \pm 40^\circ$ .

#### Preparation of A281G mutant enzyme and determination of kinetic parameters

A281G mutant of Bj Gly:CP ligase 1 was prepared by site-directed DpnI mutagenesis,<sup>22</sup> using oligonucleotides CGGCTGCGTCGQCTTCGGCATGG and

CCATGCCGAAGCCGGACGCAGCCG to introduce the desired mutation (underlined). The plasmid pET28b (Novagen) with the Bj Gly:CP ligase 1 gene cloned into NdeI and BamHI restriction sites<sup>8</sup> was used as a template. Wild-type and mutant enzymes were overexpressed in *Escherichia coli* BL21 (DE3) by overnight induction at 15°C with 1 mM IPTG in Luria-Bertani medium. His-tagged recombinant proteins were purified by affinity chromatography on Ni-NTA agarose (Qiagen) by standard procedures. Kinetic parameters for amino acid activation were determined using ATP-PPi exchange assay, performed as previously described.<sup>8</sup>

## Results and Discussions

### Characteristics of the monomeric and the dimeric Bj Gly:CP ligase 1 forms

Due to the large size of dimeric aminoacyl-tRNA synthetases, some of the previous simulations were made on a single monomer active site, with the application of harmonic restraints to atoms outside a 20 Å radius of the enzyme active site.<sup>23-25</sup> In order to recognize the structural motifs involved in the formation of the biologically active homodimeric enzyme, and to elucidate their effects on substrate binding, we modelled both, the monomeric and the dimeric enzyme forms and in our further analysis we have focused on the biologically relevant dimeric enzyme form. However, the RMSD graph clearly indicated that the monomeric protein is unstable (Fig. S2, ESI). The predominant HTH motif fluctuations observed in both, apo- and bound-forms of the monomeric enzyme have not been identified in the dimeric form, suggesting that HTH motif is involved in dimerization and protein stabilization (Fig. S3, ESI). According to the B-factor values calculated for the water molecules we were able to identify the structural water molecules (Fig. S4, ESI). Some of them are located at the interface, between the motifs 1 and 2 belonging to different subunits (for identification of motifs see Fig. S1, ESI) and through the water-mediated hydrogen bonds aid the dimer stabilization. In both, the holo- and apo-dimer, the major structural differences between the two subunits were noticed within the motif 2. During the MD simulations, the fluctuations of subunit A\_motif 2 were considerably larger than the fluctuations of subunit B\_motif 2. Upon substrate binding, this difference was lost (Fig. S5, ESI).

### The A281G mutation does not influence the Bj Gly:CP ligase 1 structure and stability

In order to elucidate the unexpected amino acid selectivity of the Bj Gly:CP ligase 1 we have studied the wild-type enzyme with both substrates, amino acid (either Ala, Ser or Gly) and ATP, simultaneously bound into the enzyme active site. Furthermore, the structures of the same type of complexes with the A281G mutant Bj Gly:CP ligase 1 were also simulated. It was found that the effect of substrates binding on the overall enzyme conformation and stability was insignificant (Fig. 1).

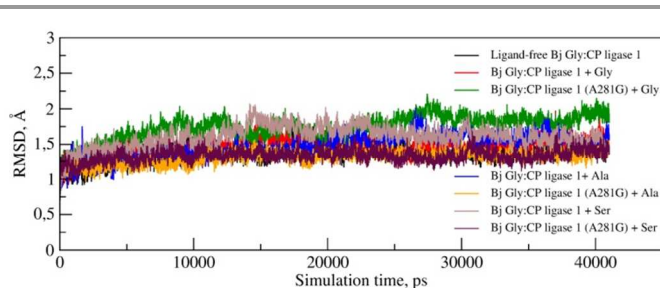
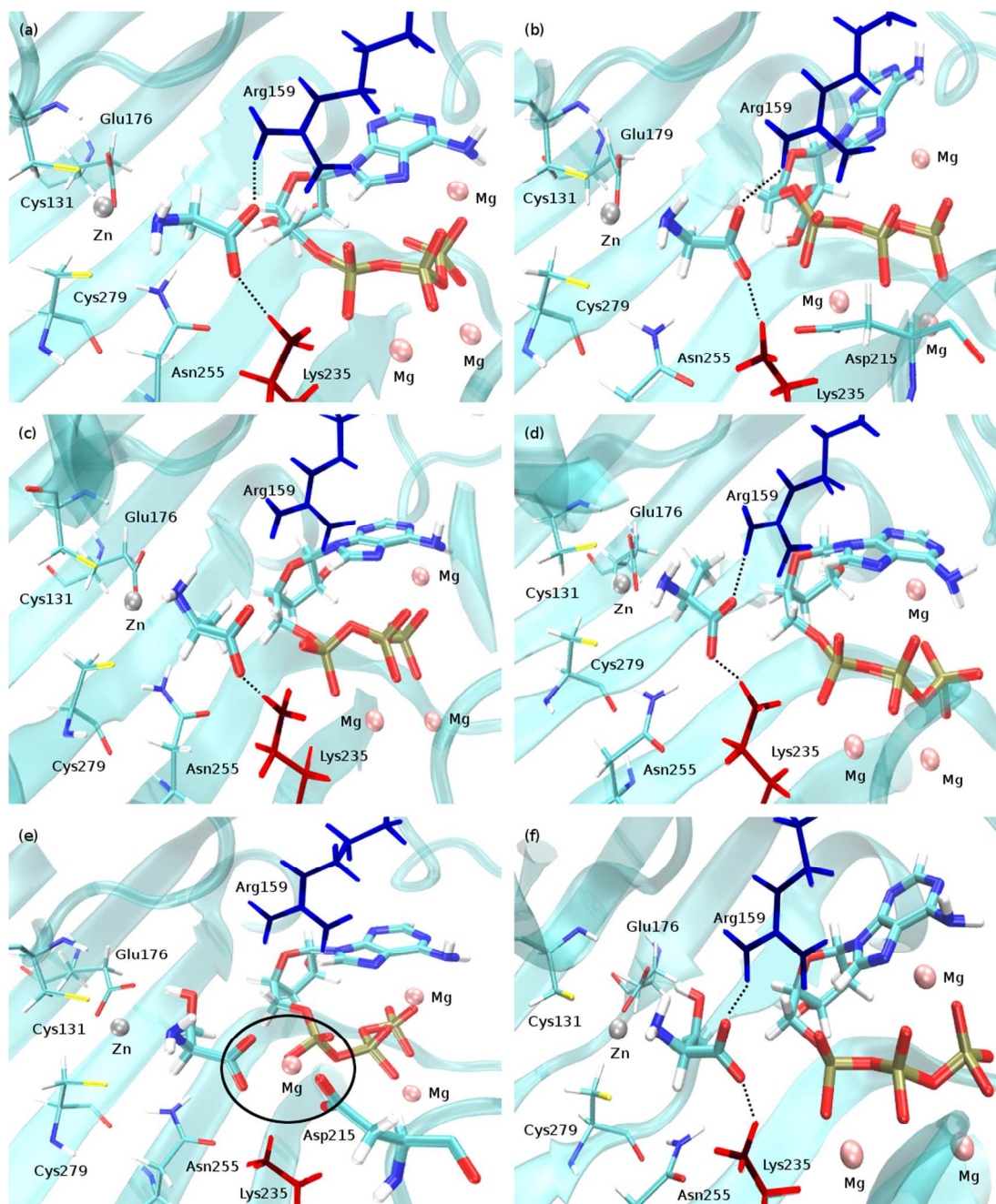


Fig. 1 The RMSD values obtained for the wild-type and A281G mutant Bj Gly:CP ligase 1 during the 41 ns MD simulations.

### The amino acid binding site

The structure of the Bj Gly:CP ligase and its A281G mutant active sites of the subunit A at the end of the 41 ns MD simulations is shown in Figure 2. In the active site of Bj Gly:CP ligase, Zn cation is tetraordinated by three conserved amino acid residues, Cys131, Glu176, Cys279 and one water molecule in a manner characteristic for the methanogenic SerRSs. In the complex, a substrate amino group replaces the water molecule in the zinc coordination sphere, keeping the metal ion coordination number unchanged. The substrate amino group is additionally stabilized by hydrogen bonding to Glu176, a residue that also coordinates the zinc cation.

In the subunit A of all complexes, the amino acid substrate remains coordinated to zinc, through its amino group, during the entire MD simulations (Fig. S6, ESI). However, the substrates have not retained their initial positions, but have reoriented in order to adopt more favourable positions. In the case of both, the wild-type and mutated (A281G) enzymes complexes with Gly, the side chains of Lys235 and Arg159 were stabilizing the substrate carboxyl group during the whole simulation (Figs. 2a and b). The carboxyl group of the substrate alanine interacted with Lys235 side chain, while the interaction with Arg159 side chain was lost (Fig. 2c). In the mutated enzyme, the alanine carboxyl group was stabilized by the side chains of Arg159, Lys235, Ser253 and Asn255 during the entire simulation (Figs. 2d and 3). In the wild-type enzyme the carboxyl group of the substrate serine together with the Asp215 side chain and the  $\alpha$ -phosphate group of ATP formed coordination sphere around the magnesium ion at the beginning of the simulation and this coordination was preserved until the end of the simulation (Fig. 2e). In comparison with the orientations of serine and of glycine in Mb aSerRS (PDB code 2CJB) and in Bj Gly:CP ligase 1 (PDB code 3MF1), respectively, this clearly represents an unproductive binding mode of serine in the active site. Namely, in such orientation the Mg cation hinders the nucleophilic attack of the serine carboxyl group to the  $\alpha$ -phosphorus atom of ATP and prevents formation of the covalent bond. In the mutated enzyme, serine is H-bonded with Lys235 during the whole simulation period and occasionally interacts with Arg159 (Figs. 2f and 3).



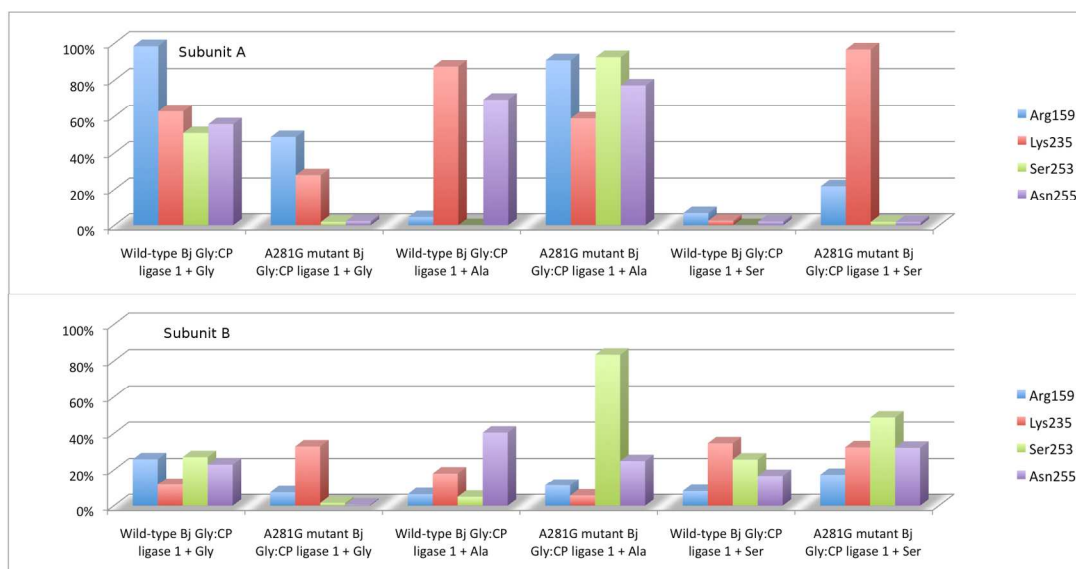
**Fig. 2** The structure of the active sites of the subunit A at the end of the 41 ns long MD simulations: (a) Wild-type Bj Gly:CP ligase 1 with glycine; (b) A281G mutant Bj Gly:CP ligase 1 with glycine; (c) wild-type Bj Gly:CP ligase 1 with alanine; (d) A281G mutant Bj Gly:CP ligase 1 with alanine; (e) wild-type Bj Gly:CP ligase 1 with serine, where the ellipse highlights the hexacoordinated magnesium cation; (f) A281G mutant Bj Gly:CP ligase 1 with serine. Black dotted lines represent the hydrogen bonds between the carboxyl group of the bound amino acid and the enzyme active site residues discussed in the text. Residues Arg159 and Lys235 are coloured in blue and red, respectively. Ser253 is not shown for clarity.

In both, the wild-type and the A281G mutated enzyme complexes with glycine, Arg159 and Lys235, each from the opposite side of the active site, hold the glycine carboxyl group in proximity to ATP  $\alpha$ -phosphate group, positioning it in the orientation favourable for the reaction (Fig. 2a and 2b). In the case of the wild-type enzyme in complex with alanine, the hydrogen bond with Arg159 was lost during the simulation (Fig. 2c). Since this hydrogen bond stabilizes

reactant in proper orientation for the nucleophilic attack, we assume that it might be important for the enzyme activity<sup>8</sup> (Table 2) towards alanine. However, in the mutant (A281G) enzyme, alanine remains hydrogen bonded to Arg159 side chain during the entire simulation time (Figs. 2d and 3). In the case of the wild-type enzyme in complex with serine, the Arg159 and Lys235 H-bonds were lost. Moreover, the Mg cation position between the serine carboxyl group

and the ATP  $\alpha$ -phosphate is unfavourable for the reaction since it disrupts the formation of the covalent bond between these two substrates (Fig. 2e). With the introduced Ala281 to Gly mutation, the enzyme active site became more spacious and it could accommodate serine in an optimal orientation for the reaction (Fig. 2f). Moreover, H-bonds between the serine carboxyl group and side chains of Lys235, as well with Arg159 could be established (Figs. 2f and 3). In contrast to subunit A, the structure of the active site in subunit B changed more drastically in all analysed cases. Substrate glycine rotated significantly so the interactions between substrate carboxylate group and the side chains of the residues highlighted as important were significantly reduced. Substrate alanine has also significantly rotated in the active site of the subunit B. In the active site of the A281G mutant enzyme, the side chain of Ser253 and hydroxyl group of ATP ribose ring interacted with the carboxyl group of the substrate alanine. In the wild-type enzyme, the non-reacting serine remained coordinated to  $Zn^{2+}$  through its amino group during the whole simulation. The side chains of Lys235, Ser253 and Asn255 periodically interacted with the carboxyl group of the substrate serine, while its side chain hydroxyl group interacted with Glu176, the Zn cation coordinating residue. In the A281G mutated enzyme, substrate serine remains coordinated to Zn cation through its amino group during the whole simulation, while its

carboxyl group interacted with the side chains of Lys235, Ser253 and Asn255. Residues of the active sites highlighted as important participate in the stabilisation and proper orientation of the substrate for the reaction. Namely, the side chains of Arg159 and Lys235 have orientations in which they interact with carboxyl group of the amino acid substrate and  $\alpha$ -phosphate group of the ATP. In some of the analysed structures, these interactions were lost during MD simulations. For instance, if the amino acid substrate rotates significantly or if Mg cation intercalates between both substrates (amino acids and ATP). Besides these two residues, side chain of Ser253 also dominantly interacted with the carboxyl groups of the glycine and alanine substrates in the active sites of wild-type and mutated enzymes, respectively. However, in all other analysed structures, the hydroxyl of the Ser253 was not oriented towards carboxyl group of the amino acid substrate and consequently could not interact with it. In order to reveal hydrogen bonds important for the enzymatic reaction, we have analysed the hydrogen-bonds network formed between the amino acid substrates (Gly, Ala and Ser) and the key active site residues, in both subunits, of the wild-type and the mutated A281G enzyme. The hydrogen-bonds occupancies (Fig. 3) as well the substrate positioning during MD simulations revealed the hydrogen bonds important for the enzyme activity.



**Fig. 3** Occupancy percentage of the H-bonds between the amino acid substrates, Gly, Ala and Ser and the key active site residues of both subunits of the wild-type Bj Gly:CP ligase 1 and its A281G mutant, determined during 41 ns of MD simulations.

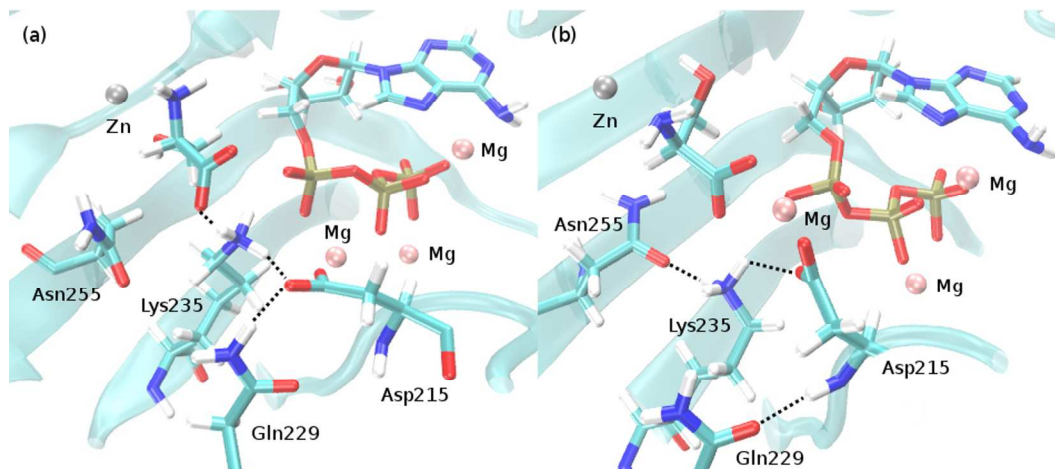
By comparing the enzyme activity (Table 2) and the hydrogen bond patterns (Fig. 3) we assumed that the H-bonds formed between the substrate and the active site residues Arg159, Lys235, Ser253 and Asn255 (Fig. 3) are important for catalysis. Indeed, in the wild type enzyme complexed with alanine, two of these four H-bonds (with Arg159 and Ser253) were lost during the simulation, while in the completely inactive complex with serine (Table 2) all these bonds were lost (subunit A) or significantly reduced (subunit B) (Fig. 3). On the other hand, in the complex of the mutated enzyme with alanine the specified bonds were present during the entire simulation stabilizing the

substrate in an orientation optimal for the reaction. It seems that the hydrogen bonds formed between the carboxyl group of the amino acid substrate and the active site residues Arg159 and Lys235 are necessary to properly orient substrate for its nucleophilic attack to ATP. Besides, the positively charged and polar side-chains groups of Arg159 and Lys235 withdraw the negative charge from the substrate carboxylate making it more suitable for the reaction. By introducing the A281G mutation in the active site of the enzyme, hydrogen bonds between the substrate alanine (and serine) and the active site residues Arg159 and Lys235 were established. Indeed, the predicted



increase of the enzymatic activity of the A281G mutant toward Ala and Ser was later on confirmed by the measurements (Table 2). It is noteworthy that the hydrogen bonds occupancy (Fig. 3.) is different within the two subunits of the wild-type and A281G mutated enzyme. This will be discussed later in *Subunits asymmetry* section.

Detailed analysis of behaviour of Lys235 during the MD simulations revealed that in the absence of the hydrogen bond between Lys235 and the substrate carboxyl group, the Lys235 side chain reoriented and the intramolecular H-bonds between Gln229, Asp215, Lys235 and Asn255 were established. As a consequence, the magnesium cation changed its position and anchored between the amino acid substrate carboxyl group and the  $\alpha$ -phosphate group of ATP (Fig. 4).



**Fig. 4** Two characteristic hydrogen bonding motifs in the enzyme active site where (a) represents the hydrogen bonds network productive for the catalysis and (b) represents the unproductive hydrogen bonds network.

Such an arrangement represents an unproductive way of substrate binding. Abovementioned amino acid triad, Gln229, Asp215, Lys235, is preserved in both Bj Gly:CP and At Ala:CP ligases. However, in methanogenic *M. barkeri* SerRS this triad is not present. Mutagenesis experiments have already confirmed the importance of Asp215 and Lys235 residues. Mutants D215A and K235A are completely inactive in both amino acid activation and the overall enzyme reaction.<sup>26</sup>

#### ATP binding site

In the enzyme active site ATP exhibits a bent conformation that is unique to class II of aaRS:  $\gamma$ -phosphate folds back over the adenine base.<sup>27</sup> Intermolecular interactions, H-bonds,  $\pi$ - $\pi$  stacking interactions and cation- $\pi$  interactions, between the adenine moiety of ATP and the amino acid residues from the binding pocket are responsible for its stabilization and positioning.<sup>28</sup> During the MD simulations the adenine base was stacked between the aromatic ring of Phe172 and the guanidinium group of Arg286, both invariant residues in motifs 2 and 3, respectively. Arg residues of motifs 2 and 3 interact with the ATP  $\gamma$ -phosphate and contribute to the positioning of ATP in the active site. Invariant Arg of motif 2 interacts with the  $\alpha$ -phosphate of ATP and participates in the stabilization of pentacoordinated transition state.<sup>29</sup>

Hydrogen bonds between ATP:N1 and Leu169:NH (motif 2), ATP:NH1 and Leu169:O, ATP:NH2 and Glu161:OE2 (motif 2) were formed during the MD simulations. In the first one, ATP acts as a proton acceptor, while, in second and third bond, ATP acts as proton donors. The  $\pi$ - $\pi$  interactions are created between ATP-adenine ring and Phe172 (motif 2). Additionally, the conjugated adenine ring interacts with three positively charged Arg residues,

159, 168 and 286 through the cation- $\pi$  interactions. Simultaneously, Arg159 makes hydrogen bonds with ATP  $\alpha$ -phosphate.

#### The role of Mg<sup>2+</sup>

Presence of three hexacoordinated Mg<sup>2+</sup> ions is characteristic for class II aminoacyl-tRNA synthetases.<sup>30</sup> Water molecules and several well-conserved residues, such as Asp and Glu, complete the hexacoordination of Mg cations. Two Mg cations have a stabilizing role bridging the  $\beta$ - and  $\gamma$ -phosphate of the ATP molecule in the active site. Third Mg cation seems to have catalytic function. It binds between the  $\alpha$ - and  $\beta$ -phosphate of ATP participating in the stabilization of the transition state by interacting with the highly charged  $\alpha$ -phosphate.<sup>29,31</sup> Magnesium cations contribute to the withdrawal of electrons from the phosphate groups, facilitating the attack of the  $\alpha$ -carboxylate group of amino acid in order to form aminoacyl-adenylate.

One of the Mg cations was initially positioned in proximity of the conserved residue Glu237 and  $\alpha$ -phosphate of ATP. The other two Mg cations bridged the  $\beta$ - and  $\gamma$ -phosphate groups of ATP, each from the opposite side. During the simulations Mg cations changed their initial positions. In the majority of cases, the Mg cation close to ATP  $\alpha$ -phosphate group ended up coordinating the carboxyl group of Glu237, one water molecule, oxygens from ATP  $\alpha$ - and  $\beta$ -phosphates as well as the carboxyl side chain of Asp215 (in the idiosyncratic LH-motif). In the case of the wild-type enzyme complexed with serine, Mg cation in proximity of ATP  $\alpha$ -phosphate ended up in between two substrates as a stable bridge between the serine carboxyl group and the ATP  $\alpha$ -phosphate oxygens. Furthermore, the Asp215 (LH-motif) carboxyl group entered the Mg coordination sphere (Fig. 2e). Mg cation, coordinated in this way,

prevents the serine carboxyl group attack on the  $\alpha$ -phosphate of ATP. As a consequence, seryl-adenylate can not be formed.

### Kinetic properties of A281G mutant of *B. japonicum* Gly:CP ligase 1

We selected the A281G mutation, as the most prominent candidate for the selectivity switch. In the wild-type enzyme, Ala281 side chain is oriented towards a substrate side chain, disabling proper accommodation of alanine and serine. The mutation of Ala281 to Gly resulted in an increase of the active site volume, allowing accommodation of alanine and serine in an orientation optimal for the reaction. Such substrate orientation, optimally positioned for the attack on the ATP  $\alpha$ -phosphate, was preserved during the simulations of the mutated enzymes. Moreover, during the simulations the hydrogen bonds between amino acid substrates and active site residues predicted as important for the enzymatic reaction were established.

Experimental data on catalytic activity of A281G Bj Gly:CP ligase 1 mutant fully support predictions based on the results of MD simulations. Wild-type enzyme has a strong preference for glycine. Alanine is only weakly activated, about 300-fold less efficient compared to glycine, but serine is not a substrate for the wild-type Bj Gly:CP ligase 1 at all<sup>8</sup> (Table 2). Mutation A281G did not affect activation of glycine; the catalytic parameters for glycine activation remained essentially unchanged compared to the wild-type enzyme (Table 2). Therefore, the mutation introduced did not perturb the architecture of the active site and the enzyme remained fully functional with regard to its cognate substrate, i.e. glycine. However, the overall specificity has changed dramatically: the relative affinity of the A281G mutant enzyme for alanine ( $K_M(\text{Ala}) / K_M(\text{Gly}) = 0.23$ ) has increased 120-fold compared to wild-type enzyme ( $K_M(\text{Ala}) / K_M(\text{Gly}) = 27$ ), and furthermore the turnover number ( $k_{\text{cat}}$ ) of the A281G mutant for alanine has increased to a value

comparable for the  $k_{\text{cat}}$  in glycine activation (Table 2). Overall, the mutant enzyme now recognizes alanine ( $k_{\text{cat}} / K_M = 4.0 \text{ s}^{-1} \text{ mM}^{-1}$ ) 3-fold more efficiently than glycine ( $k_{\text{cat}} / K_M = 1.5 \text{ s}^{-1} \text{ mM}^{-1}$ ), resulting in remarkable, roughly 1000-fold, increase in specificity of the A281G mutant for alanine over glycine, relative to the wild-type enzyme ( $[k_{\text{cat}} / K_M(\text{Ala})] / [k_{\text{cat}} / K_M(\text{Gly})] = 0.0032$ ). It is noteworthy that reversal of amino acid specificity has been achieved, since the mutant enzyme more efficiently activates alanine than glycine.

Moreover, the A281G mutation allowed the productive accommodation of serine in the active site of Bj Gly:CP ligase 1. While serine is not a substrate for the wild-type enzyme, and the parameters for serine activation could not be determined,<sup>8</sup> the A281G mutant activates serine aptly, with comparable efficiency to glycine or alanine activation (Table 2). Both  $K_M$  and  $k_{\text{cat}}$  for serine are of the same order of magnitude as catalytic parameters for alanine or glycine activation by the mutant enzyme and the catalytic efficiency ( $k_{\text{cat}} / K_M$ ) of serine activation is comparable to the catalytic efficiency of glycine activation. Given that activation of serine by wild-type enzyme is non-existent or so weak that it is below the detection limit of the assay, the increase of efficiency toward serine over glycine activation of the mutant enzyme compared to wild-type enzyme is actually far greater than 1000-fold increase in efficiency of alanine activation. The catalytic properties of mutated *B. japonicum* enzyme resemble traits of promiscuous *A. tumefaciens* Ala:CP ligase, which preferentially activates alanine, but also utilizes glycine and serine as moderately good substrates.<sup>8</sup> As already mentioned, *A. tumefaciens* Ala:CP ligase is a natural enzyme variant which harbours Gly at position 296 equivalent to Ala281 in *B. japonicum* ligase.

In conclusion, A281G mutation allowed productive binding of both alanine and serine into the Bj CP:ligase 1 active site, without any adverse effect on activation of cognate amino acid – glycine, as predicted by MD simulations.

**Table 2** kinetic parameters of amino acid activation by wild-type and mutant A281G Bj Gly:CP ligase in ATP-pyrophosphate exchange assay. Data for the wild-type enzyme are taken from Mocibob *et al.*<sup>8</sup> The values are given as arithmetic mean  $\pm$  standard error of mean (SEM). n. d. = not determined.

Substrate	Wild-type*			A281G mutant		
	$K_M / \text{mM}$	$k_{\text{cat}} / \text{s}^{-1}$	$k_{\text{cat}} / K_M / \text{s}^{-1} \text{ mM}^{-1}$	$K_M / \text{mM}$	$k_{\text{cat}} / \text{s}^{-1}$	$k_{\text{cat}} / K_M / \text{s}^{-1} \text{ mM}^{-1}$
Gly	$0.93 \pm 0.09$	$1.5 \pm 0.1$	$1.7 \pm 0.2$	$0.5 \pm 0.1$	$0.74 \pm 0.02$	$1.5 \pm 0.4$
Ala	$25 \pm 4$	$0.14 \pm 0.02$	$0.0054 \pm 0.0002$	$0.13 \pm 0.3$	$0.50 \pm 0.07$	$4.0 \pm 0.4$
Ser	n. d.	n. d.	n. d.	$0.34 \pm 0.08$	$0.57 \pm 0.05$	$1.9 \pm 0.4$

\*Data from Mocibob *et al.*<sup>8</sup>

### Subunits asymmetry

According to the results of molecular dynamics simulations, the amino acid activation step is not performed simultaneously in the both active sites of the dimer. Different behaviour of two subunits was consistently observed in all MD simulations. Besides differences in the flexibility profile between two subunits of the ligand-free enzyme (Fig. S5, ESI), asymmetry of the enzyme structure is manifested through differences in the zinc coordination

and the substrates binding (Fig. S6, ESI). In the subunit A of all analysed structures, the amino acid substrates (Gly, Ala, Ser) remain coordinated to the zinc cation during the entire simulations time. In the subunit B of the wild type enzyme, Gly and Ala were coordinated to  $\text{Zn}^{2+}$  during the first 12 ns and the first 5 ns of MD simulations, respectively. In active site B of the mutated enzyme, Gly and Ala also moved away from the Zn cation. However, Ser remains coordinated to  $\text{Zn}^{2+}$  in subunit B during the entire simulation time of both, the wild-type and the mutated (A281G) enzyme.

Coordination of Glu176 to  $Zn^{2+}$  is also different in the two subunits (Fig. S6, ESI). Besides, the hydrogen bonds occupancy is different in the active sites of subunits A and B (Fig. 3.). It is noteworthy that a certain degree of asymmetry can also be observed in the available crystal structures (PDB code 3MF1). The crystallographic subunit B lacks the amino acid sequence from 213 to 234 corresponding to the predominant fluctuating loop-helix (LH) motif, while the crystallographic subunit A contains an intact LH motif. Moreover, the active site titration experiments repeatedly showed that both active sites in the dimeric enzyme are active, and catalyse the formation of tightly bound aminoacyl-adenylate. Thus, it is unlikely that ligases display classical half-of-the-sites reactivity, like the class I dimeric TyrRS<sup>32,33</sup> where one of the catalytic subunits is completely inactive. It is more likely that the reaction proceeds with markedly different rates in the two active sites, or that the catalytic cycle progresses through some kind of ping-pong mechanism, with alternating catalytic activity of two subunits, like that described for dimeric class II HisRS.<sup>34,35</sup> The hydrogen bond occupancy (Fig. 3.) can corroborate the abovementioned postulation. Although the results of MD simulations support the asymmetric behaviour of subunits, the available experimental data do not allow for a more detailed and precise discussion of this aspect of the reaction mechanism.

## Conclusions

The objective of our work was to examine the altered substrate specificity of Bj Gly:CP ligase 1. MD simulations, together with experimental analysis, have shown that the A281G mutation strongly affects the specificity of Bj Gly:CP ligase 1. Wild-type Bj Gly:CP ligase 1 preferentially activates glycine over alanine and does not show activity towards serine. In the appropriate orientation for the reaction the methyl group of the substrate alanine clashes with Ala281 side chain. In order to minimize repulsion between the methyl groups the substrate moved away during the simulations. As a consequence, the hydrogen bond between the alanine carboxyl group and Arg159 side chain was lost. This could explain the weaker enzyme activity towards alanine compared to glycine, which can be properly accommodated in the enzyme active site. Similarly, the simulations of the wild-type Bj CP:Gly ligase 1 complexed with serine revealed an unproductive serine orientation, in which the hydrogen bonds, determined as important for the substrate stabilization, were missing. As a consequence, the  $Mg^{2+}$  was strongly coordinated by the serine carboxylate, the ATP  $\alpha$ -phosphate group and the Asp215 carboxyl during the simulations. In such an arrangement the Mg cation prevents the serine carboxyl to attack the  $\alpha$ -phosphate and hinders the enzymatic reaction. On the other hand, in the complexes with Bj Gly:CP ligase 1 A281G mutant, both substrates, alanine and serine, retained their optimal orientation during entire MD simulation time. *In vitro* kinetic assays carried out on the wild-type and the mutated Bj Gly:CP ligase 1 confirmed that A281G mutation strongly affects the substrate specificity of the enzyme. The increase in specificity of the A281G mutant for alanine over glycine, relative to the wild-type enzyme is

roughly 1000-fold, while the increase of efficiency toward serine over glycine is actually far greater. In conclusion, results of the molecular modeling results combined with the kinetic measurements presented in this study complement findings based on the structural data.<sup>8,26</sup> Multiple crystal structures of the Bj Gly:CP ligase 1 in complex with various substrates and intermediates provided snapshots of the enzyme catalysed reaction and allowed a reaction mechanism to be proposed, both for the amino acid activation<sup>8</sup> and for the transfer of activated amino acid to the prosthetic group of the carrier protein.<sup>26</sup> Presented MD simulations with strong experimental support further enhance our understanding of this peculiar family of enzymes related to aminoacyl-tRNA synthetases, by providing a dynamic view of the first stage of the reaction, unveiling in more details the importance of particular interactions and hydrogen bonds between the amino acid substrate and the active site residues. It is noteworthy that this study complements previous results in another interesting aspect: structural and functional investigations of the *B. japonicum* Gly:CP ligase 1 complexed with the cognate carrier protein led to a rational design of a chimeric enzyme that showed switched specificity toward the noncognate *A. tumefaciens* carrier protein,<sup>26</sup> whereas in this study we achieved the same for the amino acid substrate. Thus, the *B. japonicum* enzyme represents a unique system where the specificity for both the amino acid substrate and the macromolecular acceptor has been successfully remodeled. Besides, our study highlights that the minor remodeling of the active site architecture can have a significant effect on the substrate specificity, and suggests that the strategy consisting of MD method combined with mutagenesis experiment and *in vitro* kinetic assays can provide a promising approach to create improved or novel biocatalysts.

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