A new mechanism of post-transfer editing by aminoacyl-tRNA synthetases: catalysis of hydrolytic reaction by bacterial-type prolyl-tRNA synthetase

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1. Introduction

The aminoacyl-tRNA synthetases catalyze synthesis of aminoacyl-tRNAs, which serve as substrates for protein synthesis on ribosomes in a two-step reaction. In the first step, amino acids are activated by ATP to form the aminoacyl-adenylate intermediate. In the second step, the activated amino acid is transferred to tRNA. The aaRSs must recognize their cognate amino acids and tRNAs with high specificity, which is made possible by their complex structures (Giege, Sissler, & Florentz, 1998). Amino acid recognition is more difficult. Side chains of some amino acids differ only by one methyl group, which has little influence on the strength of binding to the active site (Fersht, Shindler, & Tsui, 1980; Jakubowski & Goldman, 1992); however, too many amino acid mis-incorporations may negatively affect protein function and the physiology of the organism (Lee et al., 2006). In the early stages of evolution, expansion of the genetic code (Fournier, Andam, Alm, & Gogarten, 2011) and increased requirements for accuracy in protein synthesis demanded the development of proofreading mechanisms by some aaRSs to correct mistakes that occur in the synthetic active site. The classical “double-sieve” scheme proposed by Alan Fersht long before its experimental confirmation predicted that many editing aaRSs have an additional active site responsible only for editing (Fersht, 1977). Typically, such domains ensure recognition and decylation of mischarged aminoacyl-tRNA (Fersht, 1977; Fersht & Dingwall, 1979), a process known as post-transfer editing. An alternative mechanism is pre-transfer editing, whereby erroneously synthesized aminoacyl-adenylate is hydrolyzed, usually in the synthetic active site (Dulic, Cvetesic, Perona, & Gruic-Sovulj, 2010; Wong, Beuning, Silvers, & Musieri-Forsyth, 2003).

Prolyl-tRNA synthetase (ProRS) is a class II aaRS (Cusack, Berthet-Colominas, Härtlein, Nassar, & Leberman, 1990; Eriani, Delarue, Poch, Gangloff, &
Moras, 1990). The bacterial form of ProRS is able to mischarge tRNAPro with alanine and cysteine with potentially deleterious frequency but has evolved to edit out alanine by both pre-transfer and post-transfer mechanisms (Beuning & Musier-Forsyth, 2000, 2001). Pre-transfer editing by ProRS is localized to the main active center (Wong, Beuning, Nagan, Shiba, & Musier-Forsyth, 2002) and includes a set of feasible pathways characterized by different velocities (Boyarshin et al., 2013). Post-transfer editing occurs in a dedicated active site in the INS (insertion) editing domain. The crystal structure of Enterococcus faecalis ProRS revealed for the first time the structure of the editing domain of a bacterial ProRS (Crepin, Yaremchuk, Tukalo, & Cusack, 2006). The functions of some individual amino acid residues of INS bacterial ProRSs were studied (Boyarshin et al., 2009; Kumar, Das, Hadad, & Musier-Forsyth, 2012; Wong et al., 2002), and the 2′-OH group of A76 of Ala-tRNAPro was found to be important in catalyzing the deacylation reaction (Kumar, Das, Hadad, & Musier-Forsyth, 2012). This finding supports the hypothesized substrate-assisted catalysis and suggests possible mechanisms via computational QM/MM (Kumar et al., 2012). Unfortunately, no mechanism has been experimentally confirmed (Kumar et al., 2012).

We performed comprehensive site-directed mutagenesis studies targeting the editing site of ProRS and tRNAPro variants with substitution of the 2′-OH group. Then, MD simulations and QM calculations were used to identify and study the transition-state structure and the details of the catalytic mechanism. Our experimental data, along with the results of computational investigations, strongly support the hypothesis that 2′-OH of the substrate, A76 Ala-tRNAPro, plays a key role in hydrolysis by forming an intramolecular hydrogen bond with the substrate amino acid carbonyl group. Bonding increases the electrophilic character of the carbon atom and strongly facilitates the subsequent nucleophilic attack by water molecule. Participation of a second water molecule in the process further facilitates the hydrolysis. On the other hand, the results also support the role of both substrate functional groups and the protein backbone in catalysis. In sum, this editing mechanism is significantly different from those described in the literature for class-I and class-II aaRSs (Ahmad et al., 2015; Boero, 2011; Dock-Bregeon et al., 2004; Hagiwara, Field, Nureki, & Tateno, 2010; Kumar et al., 2012; Ling, Roy, & Ibba, 2007; Tworowski, Klipcan, Peretz, Moor, & Safro, 2015).

2. Experimental procedures

2.1. In vivo expression and purification of tRNAPro

tRNAPro expression was performed in vivo in JM101 E. coli using the pBSTNAV3 vector (Meinnel, Mechulam, & Fayat, 1988) containing the lpp-promotor and rmec-terminator (the plasmid encoding tRNAPro was the kind gift of Thibaut Crepin). Three-step chromatography for tRNAPro purification was conducted with Toyopearl 650 M anion-exchange resin, HPLC on a Spherogel TSK DEAE 5PW anion-exchange column, and a Vydac C4 reverse-phase column. tRNAPro isolation and purification were performed as previously described (Boyarshin, Krikiivy, & Tukalo, 2008).

2.2. tRNAProAla in vitro expression and purification

A hybrid gene based on the E. faecalis tRNAPro gene carrying nucleotide substitutions C1G, C70T, and G72C was obtained by PCR and cloned into the pUC18 vector under control of the T7 promoter. Plasmid DNA was amplified in E. coli and purified in preparative amounts as described by Birnboim and Doly (1979). The correct template termination with CCA was ensured by cutting the DNA matrix with BstNI. Gene transcription was run for five hours using T7 polymerase with 4 mM of each NTP. After the pellet of pyrophosphate was removed by centrifugation, the tRNA transcript was purified using the DEAE-Toyopearl (Toyosoda) anion-exchange sorbent. Detailed description of the tRNAProAla isolation and purification protocols is available in our earlier articles (Boyarshin et al., 2008, 2009).

2.3. ProRS expression and purification

Enterococcus faecalis ProRS and its mutant forms were expressed in BL21(DE3)Star E. coli. The gene was ligated into the pQE70 vector under control of the T5 promoter and lac operator (the plasmid encoding E. faecalis ProRS was the kind gift of Franck Danel). After induction with 1 mM IPTG, the culture was incubated for 3.5 h at 37°C. Purification procedure included stepped salting, dialysis, anion-exchange chromatography on DEAE-Sepharose (Pharmacia), and reverse salting on Toyopearl HW60 (Toyosoda). Sample purity was verified using SDS-PAGE (Laemmli, 1970) and exceeded 95% in all cases. Protocols for E. faecalis ProRS isolation and purification are described in detail in our previous work (Boyarshin et al., 2008). Site-directed mutagenesis of the E. faecalis ProRS gene was performed by the Quick-Change (Stratagene) method (Stratagene Co., 2009). The results were verified by sequencing.

2.4. AlaRS expression and purification

Enterococcus faecalis AlaRS was expressed in BL21 (DE3)Star E. coli in a genetically engineered construction kindly gifted by Michel Arthur. Cells were lysed by ultrasonication in 50 mM tris-HCl (pH 7.5) containing 300 mM NaCl and 2 mM β-mercaptoethanol. Clarified
2.5. Aminoacylation assays

The tRNA was aminoacylated with proline in a reaction mixture containing 100 mM HEPES pH 7.0, 10 mM MgCl₂, 3 mM ATP, 0.6 mM proline, 37.6 μM 14C-labeled proline, 30 μM tRNA<sub>Pro</sub>, and 20 nM–0.2 μM ProRS<sub>Ef</sub>. The mixture was incubated at 37°C, and 10 μl aliquots were taken in 10% TCA. Precipitated Pro-tRNA was transferred onto glass microfiber filters GF/C (Whatman), washed with 50 ml of 5% TCA, and analyzed using a scintillation counter.

2.6. Preparation of 2′- and 3′-A76 modified tRNA

The tRNA CCA-terminus was cleaved in a mixture containing 50 mM tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 100 mM tRNA<sub>Pro</sub>, and 0.02 U/ml Crotalus adamanteus phosphodiesterase for 15 min at room temperature. Truncated tRNA was cleaned using phenol-chloroform extraction, re-precipitated in 8% PAAG in the presence of 7 M urea, and 20 μl aliquots were taken in 8% PAAG. Extracted product was transferred onto glass microfiber filters GF/C (Whatman), re-precipitated in 50 mM tris-HCl pH 7.5, 0.5 mg/ml BSA, 15 mM MgCl₂, 3 mM ATP, 30 μM alanine, 78 μM 14C-labeled alanine, 16 μM tRNA<sub>ProAla</sub>, and 0.6 μM E. faecalis AlaRS, then purified using phenol-chloroform extraction and re-precipitation.

2.7. Preparation of aminoacyl-tRNA substrates

(14C)Pro-tRNA<sub>Pro</sub> (WT) and its A76-derivates were prepared by aminoacylation of tRNA with proline in a reaction mixture containing 100 mM HEPES pH 7.0, 0.5 mg/ml BSA, 10 mM MgCl₂, 3 mM ATP, 0.6 mM proline, 37.6 μM 14C-labeled proline, 30 μM tRNA<sub>Pro</sub>, and 20 μM ProRS<sub>Ef</sub>. The mixture was incubated for 20 min at 37°C, extracted by phenol-chloroform and then re-precipitated.

Ala-tRNA<sub>ProAla</sub> and Ala-2′dA76tRNA<sub>ProAla</sub> were labeled by incubation for 30 min at 37°C in a reaction mixture containing 100 mM tris-HCl pH 7.5, 0.5 mg/ml BSA, 15 mM MgCl₂, 3 mM ATP, 30 μM alanine, 78 μM 14C-labeled alanine, 16 μM tRNA<sub>ProAla</sub>, and 0.6 μM E.

2.8. Deacetylation assays

Deacetylation was carried out in a mixture containing 100 mM HEPES pH 7.0, 0.1 mg/ml BSA, 10 mM MgCl₂, 2 mM DTT, 1.4–4.6 μM 14C-labeled Ala-tRNA<sub>ProAla</sub> or Ala-2′dA76tRNA<sub>ProAla</sub>, and 20 nM–6 μM ProRS<sub>Ef</sub>. Next, 2 μM pro-tRNA<sub>Pro</sub> and its derivatives were diluted to 1 μM, and 10 mM mutant H366A ProRS<sub>Ef</sub> was added. The mixture was incubated at 37°C, and aliquots were transferred onto glass microfiber filters GF/C (Whatman) pre-soaked with 10% TCA and then washed in 50 ml of 5% TCA and analyzed using a scintillation counter. Observed reaction rate constants (k<sub>obs</sub>) were calculated by dividing the steady-state rate of the reaction in μM/s, determined from the slope of the initial linear area of reaction plot, by the enzyme concentration in μM.

2.9. Computer modeling of ProRS<sub>Ef</sub> in complex with post-transfer editing substrate (Ala-tRNA<sub>ProAla</sub>)

A model of a tRNA fragment was built with Marvin Sketch software. A working model of ProRS<sub>Ef</sub> with the CCA-terminus of tRNA<sub>Pro</sub> directed to the INS domain was obtained using the HADDOCK server. A structural model of ProRS<sub>Ef</sub> in the complex with tRNA<sub>Pro</sub> was used as a template (Crepin et al., 2006). When flexible docking began, two half-flexible segments were identified: the last five bases of the tRNA 3′-terminus and five residues of each strand of linker segment connecting the INS domain with the catalytic site. Particular attention was paid to amino acid residues that affect binding of the tRNA 3′-terminus in vitro. “Conservative” protein residues were defined in the regions of protein–RNA interactions in the crystal structure to limit the search area. The results for RNA–protein docking were analyzed and evaluated using VMD based on the base orientation and the number of hydrogen bonds formed between the tRNA and the amino acid residues of the enzyme. Docking of the Ala-tRNA fragment in the INS domain was visualized in AUTODOCK using a LAMarkkian genetic algorithm. Overall, 20 docking starts were simulated. During 20 ns of free dynamics, the INS domain structure was taken every 400 ps. All possible ligand conformations were clustered by RMSD and the local energy minimum for ligand–protein binding. Selected results were analyzed in Chimera. A complex with properly oriented ligands and a sufficient number of hydrogen bonds between the purine and ligand amino group was selected for further investigation of their molecular dynamics (MD). The temporal characteristics of the complex were visualized in a MD simulation. Gromacs 4.5 software with an amber99 force field was
entirely fit to experimental inquiries. R.E.D.III was used to
determine the parameters (data on interatomic interac-
tions, partial charges) for the Ala-tRNA molecule. The
complete tRNA structure with alanine attached to ribose
O3' was simulated using the LEaP module in the Amber-
Tools software toolkit. The thickness of the water layer
surrounding the complex was equal to 10 Å, and the
ionic strength corresponded to that of a 150 mM solution
of Na+ and Cl- ions. To accelerate the calculation, the
integration time step (dt) was set to 2. The cut-off for
Van der Waals and Coulomb interactions was approxi-
mately 12 Å. Steep and Cg algorithms were used for pre-
liminary optimization of the structure. The system then
tested a series of dynamics to evaluate water distribution.
DPOSRE, which was used as a standard mechanism of
positional limitations, was replaced by COM Pulling to
ensure system stability. Thus, position restraints were
applied to all atoms through the Umbrella potential har-
monic function. During five short dynamics of 5 ps each,
the force constant gradually decreased from 500 kcal/mol
* A2 to 5 kcal/mol * A2. Free dynamics of 20 ns dura-
tion were initiated to evaluate the structural relaxation of
the tRNA complex without the aminoacyl group. The
free dynamics of the complete complex were monitored
for 5 ns. To analyze the trajectories and confirm complex
stability during dynamics testing, the g_rms and g_energy
tools were used. To investigate the mechanisms of
ligand interaction with water molecules, the g_dist tool
and visualizer PyMol 1.1. were applied.

2.10. Quantum mechanical computational methods

Computer modeling of the aminoacyl hydrolysis mecha-
nism was carried out using quantum mechanical (QM)
approaches. Density Functional Theory (DFT) calcula-
tions were performed using the B97-D functional with
Grimme's D2 dispersion correction (Grimme, 2006) and
a 6-31 g (d, p) basis set with the Gaussian 09 software
package (Frisch et al., 2009) provided by the Mississippi
Center for Supercomputing Research.

Full geometric optimization was performed for the
studied molecular systems. The starting atomic coordi-
nates for optimization procedure were obtained from the
MD trajectory at t = 2050 ps. The fragment of the enzy-
matic complex consisting of ProRS and Ala-tRNAPro
substrate was manually cut off to obtain 28 amino acids
located at the catalytic pocket and three terminal nucleo-
tide residues of the substrate. Four water molecules were
present at the active center of the reaction, in keeping
with MD data. All water molecules located outside the
active site were manually removed from the structure
used for QM modeling. In total, the system for QM cal-
culations contained 480 atoms and 4678 basis functions
for Ala-tRNAPro, with an OH group at the 2'-position of
adenosine A76 (479 atoms for the systems with 2'-deoxy
and 2'-fluoro-adenosine). The total charge of the system
was 0.

Transition-state points were localized using the Berny
algorithm and GEDIIS procedure (Li & Frisch, 2006) in
redundant internal coordinates (Peng, Ayala, Schlegel, &
Frisch, 1996) as follows: the optimized geometry of the
local minimum (pre-reaction complex) was taken and
then varied to correspond to the expected structure of the
transition state. These variations mainly involved the
geometrical position of water molecules and carbon atom
of the carbonyl group participating in a reaction. The
obtained coordinates of the atoms were used as the start-
ing point in the search for the transition state. There
were no restrictions imposed on either the geometry of
the studied system or the procedures for the TS search.

A Hessian matrix was obtained for each optimized
structure. At local minima, the Hessian matrix contained
only positive eigenvalues, whereas the transition states
were identified by one negative eigenvalue. In addition,
the structure of the transition-state vector was analyzed.
This structure confirms whether the given transition state
belongs to the studied reaction channel. Models and cal-
culated harmonic frequencies were visualized using the
molecular graphics program MaSk v. 1.3.0 (Podolyan &
Leszczynski, 2009). Atomic charges were obtained using
the Natural Population Analysis phase of Natural Bond
Orbital (NBO) analysis using NBO version 3.1 (Foster
& Weinhold, 1980).

3. Results

3.1. Mutational analysis of E. faecalis ProRS editing
domain

To date, there is no crystal structure with the misacylated
tRNA in the active site of the bacterial ProRS editing
domain. Therefore, the active site of the E. faecalis
ProRS editing domain was first studied by site-directed
mutagenesis. The amino acid residues for alanine
replacement were selected based on previous work show-
ing the location and structure of the editing (INS)
domain active site of ProRSEf and the docking model of
Pro-tRNAPro binding in the ProRSEf editing site (Crepin
et al., 2006 and Supplementary Figure S1) (Figure 1).
Among them was the highly conserved GXXXP motif,
where Gly331 is hypothesized to play an analogous role
to that of an oxyanion hole. To evaluate the post-transfer
editing activity of the obtained ProRSEf mutant forms, a
hybrid tRNAProAla carrying identity elements for both
prolyl- and alanyl-tRNA synthetases (AlaRS) was syn-
thesized using tRNAPro (Boyarshin et al., 2009). This
tRNA can be aminoacylated with alanine by means of
AlaRS and deacylated by ProRS via post-transfer
editing (Figure 2). Rates of Ala-tRNAProAla hydrolysis
by mutant forms of ProRSEf (T257A, I263A, I278A,
K279A, S280A, G331A, S332A, I333A, G334A and H366A, for alignment see Supplementary Figure S2) were compared to the rate of hydrolysis of the substrate by the wild-type enzyme (Table 1). The most pronounced effect was observed when the highly conserved amino acid residue Lys279 was replaced on alanine, resulting in 2006.3-fold decrease in post-transfer editing activity. An appreciable effect was also observed for mutations at residues Ile278, Gly331, Ile333, and His336, which reduced post-transfer editing activity of the enzyme 12.8, 6.7, 54.7, and 4.4-fold, respectively. The other mutations had no significant effect on ProRS post-transfer editing activity (Table 1). In summary, the mutagenesis study provided experimental support for identification of a putative deacylase active site of ProRS where absolutely conserved Lys279 and two amino acids from GXXXP motive, conserved Gly331 and highly conserved Ile333, have some functional role.

Table 1. Ala-tRNA<sub>ProAla</sub> hydrolysis by WT ProRS<sub>Eff</sub> and its mutant forms.

| K279A | S280A | G331A | S332A | I333A | G334A | H366A |
|-------|-------|-------|-------|-------|-------|-------|
| Δk<sub>obs</sub>, s<sup>-1</sup> | 0.383 ± 0.047 | 0.353 ± 0.069 | 0.157 ± 0.063 | 0.030 ± 0.005 | 0.190 ± 0.006 | 0.007 ± 0.005 |
| Loss of activity, fold | 1.0 | 2.4 | 6.7 | 12.8 | 2006.3 | 4.4 |

Figure 1. Ribbon representation of ProRS<sub>Eff</sub> INS domain. Putative active-site residues investigated by mutagenesis are shown in color.

Figure 2. Time course of alanyl-tRNA<sub>ProAla</sub> deacylation by 30 nM ProRS<sub>Eff</sub> (■) and without enzyme (●).
The role of Lys279 can be explained on the basis of the model proposed earlier (Crepin et al., 2006), in which this conserved lysine interacts with the phosphate of A76. This interaction may be critical to positioning the 3'-end of the tRNA<sub>Pro</sub> in the editing active site of ProRS. On the other hand, Lys279 is unlikely to participate directly in catalysis due to its distant localization from the bond to be hydrolyzed.

3.2. Modification of 2'-OH A76 tRNA is critical for enzymatic deacylation

The A76 2'-hydroxyl group is the proximal polar group to the amino acyl ester bond. A version of its role in deacylation has been presented previously (Kumar et al., 2012b). Therefore, in the present work, the 2'-hydroxyl group of A76 tRNA<sub>Pro</sub> was considered a candidate for playing direct catalytic role in this process. To establish its role, the 2'-dA76-derivative of tRNA<sub>ProAla</sub> was synthesized using enzymatic approaches and then aminoacylated by <i>E. faecalis</i> AlaRS. The obtained Ala-2'-dA76-tRNA<sub>ProAla</sub> completely lost the capacity for deacylation in the presence of ProRS<sub>Ef</sub> (Figure 3(A)). Moreover, the enzyme appeared to protect the mischarged tRNA against spontaneous hydrolysis (Figure 3(B)). The last point indicates the effective binding of the editing substrate to the active site of the editing domain, which in turn suggests that the affinity is not significantly affected by 2'-OH substitution. The rate of cleavage of modified Ala-tRNA in the absence of the enzyme was also dramatically lower (Figure 3(C)). These data demonstrate the crucial role of 2'-OH A76 in Ala-tRNA editing by ProRS<sub>Ef</sub>. However, its catalytic function remains...
unknown because it was impossible to obtain kinetic parameters of this reaction experimentally. The protection of the substrate against non-enzymatic hydrolysis may indicate that the ester bond becomes inaccessible to water, without which the reaction is impossible.

To obtain more informative data regarding the role of the 2'-OH of tRNA in the mechanism of hydrolysis, we used the ability of the mutant form of ProRS H366A to deacylate Pro-tRNAPro (Wong et al., 2002). Pro-tRNAPro, Pro-2'-dA76-tRNAPro, and Pro-2'-F-A76-tRNAPro were prepared and used for the deacylation reaction (Figure 4), making it possible to quantify kinetic parameters of product hydrolysis (Table 2).

The rate of Pro-2'-dA76 tRNAPro hydrolysis appeared to be 600-fold lower than that of Pro-tRNAPro hydrolysis. Enzymatic hydrolysis of Pro-2'-dA76 tRNAPro is 8.4-fold faster than non-enzymatic hydrolysis. Thus, despite the persistence of the access of water to the ester bond, removal of the 2'-OH drastically affects the catalytic function of the editing domain. The rate of Pro-2'-F-A76 tRNAPro hydrolysis is approximately 150-fold lower than that of Pro-tRNAPro (Table 2). Despite its polar properties, a fluorine group at the 2'-position was unable to mimic the hydroxyl functions. The most obvious difference between these chemical substituents is the inability of the fluorine group to donate a hydrogen bond (Nordin & Schimmel, 2003). Finally, we can assume that the 2'-OH in post-transfer editing forms hydrogen bonds with certain functional groups and induces a particular conformation of the substrate, accelerating hydrolysis of the ester bond.

### Table 2. Hydrolysis of WT Pro-tRNAPro and its 2'-d and 2'-F derivatives by ProRSEf.

|                      | WT       | 2'-F         | 2'-d         |
|----------------------|----------|--------------|--------------|
| Enzymatic hydrolysis | k<sub>obs</sub> s<sup>-1</sup> | 0.246 ± 0.037 | 0.175 × 10<sup>-3</sup> ± 0.024 × 10<sup>-3</sup> | 0.422 × 10<sup>-3</sup> ± 0.052 × 10<sup>-3</sup> |
| Loss of activity, fold | 1        | 150          | 600          |
| Non-enzymatic hydrolysis | k<sub>obs</sub> s<sup>-1</sup> | 0.166 × 10<sup>-3</sup> ± 0.037 × 10<sup>-3</sup> | 0.964 × 10<sup>-4</sup> ± 0.174 × 10<sup>-4</sup> | 0.503 × 10<sup>-4</sup> ± 0.080 × 10<sup>-4</sup> |
| Loss of activity, fold | 1        | 1.72         | 3.3          |

3.3. **MD simulation of the complex of ProRSEf with mis-aminoacylated tRNAPro**

To better understand the molecular mechanisms of the editing reaction, a structural model of completely solvated ProRSEf in complex with Ala-tRNAPro was constructed using MD simulations. This is an alternative from studied in the literature, with a truncated substrate analog of Ala-tRNA, 5'-CCA-Ala, docked into of the free INS domain of ProRS (Kumar et al., 2012a, 2012b). In our opinion, the full-molecule complex model allows us to obtain a simulation that maximally takes into account the influence of the tRNA binding mode on substrate positioning. To obtain a complete structure that fits all experimental requirements, protein–nucleic acid and protein–ligand docking analysis was performed. The free energy of amino acids in the INS domain was estimated using MD simulations.
determined using the Swiss-model server (Atomic Non-Local Environment Assessment graph) (Arnold, Bordoli, Kopp, & Schwede, 2006). Negative values and green color in the diagram (Supplementary Figure S3) indicate the optimal values of amino acid energy. The location of the aminoacyl-tRNA fragment in the middle of editing domain was predicted using AUTODOCK. Figure 5(A) and B shows how the 3′-end of tRNAPro binds in extended active site in the editing domain (INS). This pocket overlaps well with the conformation predicted for the ProRSEf putative deacetylase active site (Crepin et al., 2006), which includes such functionally important elements as the GXXXP motif and conserved lysine (Lys279 in ProRSEf). In this model, Lys279 interacts with the phosphate group of A76 tRNAPro. This interaction is consistent with the X-ray structure of apo-ProRSEf, where Lys279 is bound to a solvent SO42− moiety (Crepin et al., 2006). Moreover, in our model, Lys279 interacts with the O2 of C75 and the 2′OH group of C74, promoting bending of the CCA end of the tRNA. This model differs from that of Kumar et al. (Kumar et al., 2012a), where the CCA has an extended conformation and the A76 form stacks with C75. It is noteworthy that, in our model, adenine is rotated by the N3 position to the alanine moiety, while in the model of Kumar et al., it is rotated to the phosphate group of A76. Another stable binding interaction between INS domain and CCA-Ala involves the adenine base of A76. The modeled complex shows three favorable hydrogen bonds: two between the N6 of A76 and carbonyl of Glu316 and backbone carbonyl of Leu282 and one between the N7 of A76 and carbonyl of Glu316. A76 also has a stacking interaction with Phe330. C74 also forms H-bonds with carbonyl of Asp299, and C75 forms an H-bond with the N7 of A76.

The substrate alanine moiety binds in the hydrophobic cleft in the ISN domain. Two H-bonds form between the amine group of the substrate alanine and the main chain carbonyls of residues Gly331 and Ile333. The methyl side chain of the alanine substrate binds in hydrophobic pocket described by Kumar et al. (2012a) and formed by Ile263, Ile277, His366 and to some extent by Val266 (Supplementary Figure S4).

In our model, an alanine residue is linked to the 3′ hydroxyl group of A76 in accordance with the experimental data showing that 3′-OH is the primary site of tRNAPro aminoacylation. However, some aaRSs editing mechanisms involve catalytic transacylation before aminoacyl removal (Nordin and Schimmel, 2002; Tworowski et al., 2015). To examine the possibility that this pathway functions in a proline system, we made a docking model with Ala-2′-A76. Notable differences in the Ala-3′-A76 and Ala-2′-A76 binding modes include the sharp turn of adenine base and alanine moiety, resulting in steric clashes, and loss of interactions with the protein. This steric dissimilarity is not surprising, specifically because of ribose conformation differences between regioisomers, as shown on 2′- and 3′-anthraniloyl adenosine by NMR (Acharya, Nawrot, Sprinzl, Thibaudeau, & Chattopadhyaya, 1999). Here-with, it is unlikely that transacylation could promote such rearrangements.

Detailed analysis of the MD trajectory using VMD revealed that the H-bonds between the above-mentioned amino acids of the ISN domain and the ligand atoms are sufficiently long-lived. All possible water molecules surrounding the C-group carbon and amino groups during MD were identified using the g_dist command. This command provided information concerning the number of molecules in the system, as well as the apparent time of contact location. According to these data, molecules localized near these groups for a period of time insufficient for the reaction to proceed were eliminated. The remaining molecules were evaluated for the position of hydrogen atoms and angle of atom approximation using the PyMol package with Measurement. Thus, MD frames where two water molecules formed a necessary angle of 104–105° at a distance of 3.5 Å from the carbon atom and H-bonds with protein environments were selected (Figure 5(B)). Such configurations were stored for 50 ps, while the alanine moiety was also in advantageous position to be attacked. Interestingly, these water molecules are also present in the crystal structure of ProRSEf as W2068 and W2063, respectively (Crepin et al., 2006). Thus, these two water molecules were included in the

![Figure 6. Optimized structure of the ProRSEf editing active site with bound Ala-A76 substrate and the water molecules (W1, W2, and W3) trapped in its proximity. Only some amino acid residues directly involved in hydrolysis are shown.](image-url)
quantum mechanics subsystem in view of their potential activity as nucleophilic species in hydrolysis.

3.4. QM calculations of deacylation

To investigate a possible mechanism of ProRS$_{Ef}$ post-transfer editing, we employed QM methods in conjunction with MD. Atom coordinates for full geometry optimization were taken from the MD trajectory at approximately 2 ns after the beginning. During MD, the spatial distribution of water molecules trapped in proximity of the active site allow them to play an active role in the reaction (Figure 6).

The molecular system for QM calculations was a fragment of enzymatic complex containing ProRS and the Ala-tRNA$_{Pro}$ substrate. The system contained 480 atoms (28 amino acids and 3 nucleotides) for “normal” Ala-tRNA$_{Pro}$ with 2'-OH group at terminal nucleotide, or 479 atoms for 2'-deoxy and 2'-fluoroadenosine analogues.

According to the results, hydrolysis of Ala-tRNA$_{Pro}$ directly involves two water molecules. A number of amino acid residues of the enzyme were identified as assisting in hydrolysis. Full details of the reaction profile, including the structure of transition state, are shown in Figure 7, and the energetic parameters of the reaction are presented in Table 3.

Figure 7. Mechanism of alanyl-tRNA$_{Pro}$ hydrolysis by ProRS$_{Ef}$. (A) Pre-reaction complex; arrow indicates the direction of nucleophilic attack by the water molecule; dashed lines – hydrogen bonds, (B) Transition state; hashed lines – bonds in the TS; arrows indicate bond formation/cleavage from TS to reaction products, (C) Parameters of transition state: numbers indicate bond lengths (Å), $q$(H$_3$O) – total charge on H$_3$O fragment and (D) Reaction products.
Table 3. Energetic parameters of the aminoacyl hydrolysis reaction profile for the systems containing terminal adenosine, 2′-fluoro- and 2′-deoxyadenosine in the Ala-tRNA^Pro substrate.

| 2′-Substituent | Activation energy, kcal/mol* | ΔE_{act}** |
|----------------|-----------------------------|------------|
| OH             | 18.57                       | 0          |
| H              | 31.06                       | 12.49      |
| F              | 25.73                       | 7.16       |

*the difference between full energy of the transition state and that of the pre-reaction complex.
**the difference between the activation energies of 2′-H (2′-F) and 2′-OH derivatives.

The key role of the 2′-hydroxyl group of A76 in hydrolysis was strongly supported by DFT calculations. The reaction starts with the formation of the intramolecular hydrogen bond between 2′-OH and a carbonyl group of substrate amino acid. To achieve this, the 2′-hydroxyl group and 3′-amino acid residue rotate to find a conformation where OH can easily coordinate the carbonyl group, forming a stable seven-membered cyclic structure. That change increases the electrophilicity of the C=O carbon and thus strongly facilitates the nucleophilic attack of the water molecule.

Next, two water molecules at the active site approach the carbonyl group of the amino acid to be cleaved off. One of them (W1) resembles a coordinated molecule in the apoenzyme crystal structure and in our docking model. This water molecule is poised to attack the electrophilic carbonyl group to hydrolyze the ester bond. This water molecule forms H-bonds with the Ile333 carbonyl oxygen and with the second, non-attacking H2O molecule (W2), which, in turn, forms an H-bond with a carbonyl oxygen atom of the Ile278 residue. Coordination of the proton originating from the W1 molecule with Ile333 carbonyl remains intact during the whole process. Thus, the pre-reaction complex is formed (Figure 7(A)).

At this point, the protonated amino group of 3′-alanine (NH3+) forms two H-bonds. The first bond is with the Gly331 carbonyl, whereas the second N-H proton coordinates with the phosphate anion via the third water molecule W3 (in other words, the phosphate forms a bridge with the alanine NH3+ group via this water molecule). W3 does not directly participate in the chemical reaction, so it will not be further mentioned, but its coordinating role remains unchanged during hydrolysis. Importantly, the Ala residue is conformationally fixed by the set of H-bonds, restricting its degrees of freedom and further facilitating hydrolysis.

Then, W1 attacks the alanine C=O group and a new C–OH bond starts to form in the transition state (TS, Figure 7(B)). At the same time, the second proton of this water molecule begins transferring to the 3′-O ribose oxygen. The distance between this proton and oxygen atom of W1 molecule (1.918 Å, see Figure 7(C)) indicates that this O–H bond is already almost broken in the TS. The second water molecule actively participates in the transfer of this proton by forming a strong H-bond with it during its move from W1 to the 3′-O ribose atom (Figure 7(B)). Binding of W2 to this proton results in the formation of an H3O moiety with O–H bonds of almost equal lengths (1.019–1.040 Å) and a positive charge of +0.701 (Figure 7(C)). Thus, this molecular fragment formed in the TS can be considered a quasi-hydroxonium cation with acidic character.

The effect of the 2′-OH group is greatly enhanced in the transition state, as the H-bond formed by this hydroxyl with the Ala carbonyl group becomes much stronger in the TS than in the pre-reaction complex (bond length decreases from 2.016 to 1.651 Å, while the calculated bond order increases from 0.002 to 0.161). This strong hydrogen bond is one of the key determinants of the reaction mechanism.

In the TS, the non-attacking water W2 coordinates with two amino acids of the Ile278–Lys279–Ser280 fragment: one water proton forms an H-bond with the serine OH group, whereas the second proton forms an H-bond with the isoleucine carbonyl oxygen. This water molecule is thus spatially fixed, which is very important for the subsequent steps. At the same time, the NH3+ group of the 2′-Ala residue in the TS forms an additional H-bond with a His366 nitrogen. This spatial arrangement, a three-centered coordination of the alanine amino group (W3 water, Gly331, and His366) is stable and remains conserved until the end of the process. According to QM calculations, similar transition states are also formed for the substrates containing 2′-deoxy- and 2′-fluoro-adenosine. However, the corresponding activation energies are much higher (see Table 3).

From the TS, the reaction proceeds readily to the cleavage of the Ala residue from the ribose moiety. Proton transfer to the O-3′-ribose oxygen is accompanied by the cleavage of the O-2′–C=O ester bond. At the end of the hydrolysis, the adenosine nucleoside is separated from the Ala amino acid (Figure 7(D)), which is still coordinated with W3, Gly331, and His366 via its protonated amino group. The alanine carboxylic group forms two H-bonds; the OH group is still coordinated with Ile333, whereas a carbonyl oxygen forms a new H-bond with the 3′-OH group of adenosine. At the same time, water molecule W2 remains bound to the Ile278 and Ser280 residues via hydrogen bonds. In addition, its oxygen atom forms a new H-bond with the 2′-OH group of adenosine. Then, the alanine molecule leaves the reaction site.
4. Discussion

A recently proposed tRNA-assisted mechanism of post-transfer editing suggests a role of the 2′-OH or 3′-OH groups of A76 tRNA in activation of the nucleophilic water molecule to initiate hydrolysis in PheRS (Ling et al., 2007; Tworowski et al., 2015), ProRS (Kumar et al., 2012b), LeuRS (Boero, 2011; Hagiwara et al., 2010) and archaeal ThrRS (Ahmad et al., 2015). One mechanism of editing for ProRS ef (Kumar et al., 2012b) assumes a critical role in catalysis for the Gly261 backbone carbonyl (which corresponds to Lys261 in E. coli ProRS). It is thought to be involved in shuttling a proton from the catalytic water to the 3′-O of A76. Unfortunately, the proposed mechanism has not been convincingly supported by experimental results, as the Lys261 deletion mutant E. coli ProRS shows a defect in substrate binding (see Figure 6 of Kumar et al., 2012b) and as a result showed, a dramatic loss in deacylation activity. To elucidate the mechanism of post-transfer editing of ProRS ef and to gain a qualitative understanding of the transition-state structures and energetics of the catalytic reaction, we performed detailed density-function-theory-based computational studies in conjugation with MD simulations of the full ProRS ef in complex with Ala-tRNA Pro, comprehensive site-directed mutagenesis and tRNA-modification studies.

Kumar et al. (Kumar et al., 2012b) used very limited QM part, with only 7–10 amino acid residues and one terminal adenosine (or only the ribose fragment, depending on the investigated reaction pathway), in their calculations of hydrolysis reaction mechanisms. It was thus not possible for them to identify all amino acid residues participating in the catalytic process. Moreover, different sets of functional residues were considered for different reaction mechanisms, disallowing direct comparison of the energetic parameters of possible reaction channels. Calculations based on the hybrid QM/MM approach were limited to the potential energy surface scans of theoretically proposed reaction mechanisms. In contrast, our model was more comprehensive and contained three terminal nucleotide residues of tRNA and all 28 amino acid residues of the catalytic pocket including all residues that immediately surround the substrate. This model was used for QM calculations of all predictable reaction mechanisms. The approach used allowed us to perform direct identification of the transition state of the most probable reaction pathway.

These results suggest a new mechanism of hydrolysis of misacylated Ala-tRNA Pro. The main role of 2′-OH A76 tRNA in the post-transfer editing mechanism we propose is to ensure intramolecular interaction through redistribution of the electron density, and it appears to be crucial for catalysis. Importantly, we were able to identify the transition state of the enzymatic hydrolysis reaction. This mechanism is comparable with that proposed by Bruice and Fife (1962) for non-enzymatic ester hydrolysis, where the adjacent hydroxyl donates a hydrogen to the oxygen of the tetrahedral intermediate. Thus, in contrast to the findings of previous studies, the attacking water molecule is not activated by free hydroxyl group of A76 of the misacylated tRNA, but by the main chain of Ile333 of the enzyme and the second water molecule. This activation appears to be completely insufficient for fast hydrolysis in the absence of binding between the 2′-OH and the carbonyl group. Moreover, this intramolecular bond is necessary not only for enzymatic hydrolysis but also for spontaneous hydrolysis of Ala-tRNA Pro in aqueous solution, as the rate of non-enzymatic hydrolysis of the aminoacyl group is reduced by several orders of magnitude in the absence of such a bond. Interestingly, such an intramolecular interaction was also recently suggested in the editing pathway for PheRS, which gives rise to the cyclic (2′,3′-ortho) intermediate (Tworowski et al., 2015).

The results of our QM calculations suggest that two water molecules present at the active site actively participate in the hydrolysis of Ala-tRNA Pro (an additional water molecule, W3, coordinates the alanine amino group and phosphate and is not directly involved in hydrolysis). The key starting point of the reaction is a coordination between the ribose 2′-OH proton and a C=O group of the alanine residue, which results in the formation of an intramolecular H-bond that increases an electrophilic character of a carbon atom, strongly facilitating the subsequent nucleophilic attack of the water molecule. This type of coordination is impossible in the case of either 2′-deoxyadenosine or 2′-fluoro adenosine lacking a 2′-hydroxy group, which explains to a great extent the sharp decrease in hydrolysis rates observed for corresponding modified tRNAs. The significant difference between the calculated activation energies for compounds containing OH, F, and H substituents at the C-2′ position of ribose strongly supports this conclusion. The corresponding DFT values are 18.57, 25.73, and 31.06 kcal/mol, i.e., the activation energies for 2′-F and 2′-H substrates are, respectively, 7.16 and 12.49 kcal/mol higher than those of the “normal” substrate (Table 3). The higher rate of hydrolysis of the 2′-fluoro analog compared with 2′-deoxyadenosine is due to the electronic factor, namely the strong electron-withdrawing effect of a fluorine substituent at the β-position to the acyl group to be cleaved off. Finally, the results of our QM calculations fit well with our biochemical results on the rate of Pro-tRNA Pro, Pro-2′-dA76-tRNA Pro and Pro-2′-F-A76-tRNA Pro hydrolysis (Table 2). The first water molecule, W1, performs a nucleophilic attack at the electrophilic carbonyl center of the substrate. This water molecule forms two H-bonds, one
with a carbonyl oxygen atom of Ile333 and one with the second water molecule, W2. In the transition state (Figure 7(B)), W2 partially abstracts the proton from the attacking water molecule to form a quasi-hydroxonium cation. This deprotonation increases the reactivity of W1 due to the formation of more nucleophilic species. Thus, W2 acts here as a base, and a hydroxyl quasi-anion produced by W1 attacks the electrophilic carbonyl group of the aminoacyl residue to be cleaved off. Thus, W2 can be considered a moiety that activates the attacking water, W1, by partial deprotonation. At the same time, the W2 molecule in the TS helps to transfer a proton from W1 to the 3′-O ribose atom, facilitating the cleavage of alanine from adenosine. Thus, the reaction involves two functionally very different water molecules, W1 and W2, the first one being a reactive nucleophilic species and the second an assisting moiety that acts first as a base and later as a conjugated acid. The role of the assisting water here is similar, for example, to that of histidine in the active centers of many hydrolytic enzymes: the histidine residue accepts a proton in the first step of hydrolysis (as a base) and then donates it in the second step (as an acid). From this point of view, we can consider Ala-tRNAPro hydrolysis to proceed via the general acid-base catalysis mechanism.

The crucial role of the W2 molecule in hydrolysis was additionally supported by QM calculations of a set of possible reaction mechanisms involving only one water molecule. A one-water process was energetically much less favorable: in this case, we were unable to find any reaction profile with an activation energy below 40 kcal/mol, much higher than the energy required for the two-water mechanism.

The role of structural elements of the enzyme active site cannot always be shown with a high degree of certainty, as in the case of the 2′-OH group of tRNA. Nevertheless, the role of certain amino acid residues in the editing reaction was clearly established. Lys279 appeared to be the most important of these. According to the MD simulation data, Lys279 is not included in the active center of the editing domain. However, it interacts with the CCA-end of the edited Ala-tRNAPro, binding it and orienting it in the active site. Moreover, our results are consistent with previously published data on its role in E. coli ProRS and YbaK editing (Kumar, Das, Hadad, & Musier-Forsyth, 2013; Kumar et al., 2012a). Furthermore, this interaction is likely to affect the conformation of the Ile278 and Ser280 amino acid residues that neighbor Lys279 in the polypeptide chain. Despite the participation of the serine 280 OH group in coordination of the non-attacking water molecule (W2), its substitution by alanine has little effect on the deacylation activity of ProRSeF (2-fold decrease). This effect can be explained by our molecular modeling/dynamic studies as a compensatory effect through coordination of the W2 water molecule with a carbonyl oxygen atom of Ile357 instead of the Ser280 hydroxyl.

In the proposed mechanism of hydrolysis, the main chain carbonyls of Ile333 and Ile278 coordinate two water molecules participating in hydrolysis, thus allowing proton transfer. Mutations in I333A and I278A resulted in a marked decrease in ProRSeF editing activity (by approximately 55 and 13-fold, respectively), confirming their role in the proposed mechanism of hydrolysis. Participation of a protein main-chain carbonyl in catalysis has also been established for several other enzymes that catalyze aminoacyl-tRNA hydrolysis. These enzymes include a free-standing YbaK protein in which alanine substitutions of Gly110 and Gly131 resulted in 28- to 36-fold decreases in deacetylation efficiency of enzyme (So et al., 2011). In the structurally distinct editing domain of archaeal ThrRS, a crystallographic water molecule, which is activated by the 2′-OH of the analog at the transition states, is presumably stabilized by the oxanion hole formed by the main-chain nitrogens of Ala82 and Phe81 (Ahmad et al., 2015).

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| aaRSs | aminoacyl-tRNA synthetases; |
| AlaRS | alanyl-tRNA synthetase; |
| DEAE-Sepharose | O-(diethylaminoethyl)-Sepharose; |
| DFT | density functional theory; |
| DTT | dithiothreitol; |
| INS | prolyl-tRNA synthetase insertion domain; |
| IPTG | isopropyl β-D-1-thiogalactopyranoside; |
| MD | molecular dynamics; |
| MM | molecular mechanics; |
| PAAG | polyacrylamide gel; |
| PEI | polyethyleneimine; |
| ProRS | prolyl-tRNA synthetase; |
| ProRSeF | Enterococcus faecalis prolyl-tRNA synthetase; |
| QM | quantum mechanics; |
| TCA | trichloroacetic acid; |
| TS | transition state |

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