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Rational design of aminoacyl-tRNA synthetase specific for *p*-acetyl-L-phenylalanine

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ABSTRACT

The *Methanococcus jannaschii* $tRNA^{Tyr}$/tyrosyl-tRNA synthetase pair has been engineered to incorporate unnatural amino acids into proteins in *Escherichia coli* site-specifically. In order to add other unnatural amino acids into proteins by this approach, the amino acid binding site of *M. jannaschii* tyrosyl-tRNA synthetase need to be mutated. The crystal structures of *M. jannaschii* tyrosyl-tRNA synthetase and its mutations were determined, which provided an opportunity to design aminoacyl-tRNA synthetases specific for other unnatural amino acids. In our study, we attempted to design aminoacyl-tRNA synthetases being able to deliver *p*-acetyl-L-phenylalanine into proteins. *p*-Acetyl-L-phenylalanine was superimposed on tyrosyl in *M. jannaschii* tyrosyl-tRNA synthetase–tyrosine complex. Tyr32 needed to be changed to non-polar amino acid with shorter side chain, Val, Leu, Ile, Gly or Ala, in order to reduce steric clash and provide hydrophobic environment to acetyl on *p*-acetyl-L-phenylalanine. Asp158 and Ile159 would be changed to specific amino acids for the same reason. So we designed 60 aminoacyl-tRNA synthetases. Binding of these aminoacyl-tRNA synthetases with *p*-acetyl-L-phenylalanine indicated that only 15 of them turned out to be able to bind *p*-acetyl-L-phenylalanine with reasonable poses. Binding affinity computation proved that the mutation of Tyr32Leu and Asp158Gly benefited *p*-acetyl-L-phenylalanine binding. And two of the designed aminoacyl-tRNA synthetases had considerable binding affinities. They seemed to be very promising to be able to incorporate *p*-acetyl-L-phenylalanine into proteins in *E. coli*. The results show that the combination of homology modeling and molecular docking is a feasible method to filter inappropriate mutations in molecular design and point out beneficial mutations.

Introduction

Most known organisms encode the same common 20 amino acids as building blocks, from which almost all proteins are synthesized. The side chains of these common amino acids comprise a limited number of functional groups. To overcome this natural limitation, an in vivo biosynthetic method has been established. By adding new components to the translational machinery of *Escherichia coli*, one could site-specifically incorporate a desired unnatural amino acid into proteins in vivo. This new component involve the generation of an engineered *Methanococcus jannaschii* (*M. jannaschii*) $tRNA^{Tyr}$/tyrosyl-tRNA synthetase (TyrRS) pair, which is orthogonal to its counterparts for the 20 amino acids. The orthogonal aminoacyl-tRNA synthetase (aaRS) aminoacylates only the orthogonal tRNA with the desired unnatural amino acid, and this acylated tRNA delivers this amino acid to the ribosome only in response to a non-coding codon, TAG [1].

The *M. jannaschii* TyrRS need to be mutated to add other unnatural amino acids into proteins by this approach. Usually the crystal structure of *M. jannaschii* TyrRS is used to choose residues in close proximity to the phenyl ring of substrate tyrosine (Tyr). The chosen residues will be randomly mutated to generate an aaRS library. After several rounds of experimental screenings, some clones will be chosen for their being able to incorporate unnatural amino acid efficiently and selectively into proteins. More than 25 unnatural amino acids have been incorporated into proteins in *E. coli* by this approach. These include amino acids that can be photocrosslinked, glycosylated, and other chemically reactive amino acids containing keto, alkene or alkyne groups, redox active amino acids and fluorescent amino acids [2–8].

The ability to alter the genetically encoded proteins with new amino acids would significantly enhance our ability to manipulate the structures and functions of proteins and even living organisms themselves. Taking the keto group for example, the keto group participates in large numbers of reactions from addition reactions

Abbreviations: aaRS, aminoacyl-tRNA synthetase (s); *M. jannaschii*, *Methanococcus jannaschii*; TyrRS, tyrosyl-tRNA synthetase; Tyr, tyrosine; OMeTyr, *O*-methyl-L-tyrosine; OMeTyrRS, *M. jannaschii* TyrRS mutant specific for *O*-methyl-L-tyrosine; pAcPhe, *p*-acetyl-L-phenylalanine; MOE, Molecular Operating Environment.

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to aldol condensations in organic chemistry. Moreover, the reactivity of keto group allows it to be selectively modified in the presence of other amino acid side chains [9–11].

To understand the molecular basis of the specificity of the wild-type and mutant orthogonal TyrRS, the crystal structures of *M. jannaschii* TyrRS [12] and a mutant TyrRS specific for *O*-methyl-L-tyrosine (OMeTyrRS) [13] were determined. These provided an opportunity to design aaRS specific for other unnatural amino acids.

The overall structure of *M. jannaschii* TyrRS is similar to that of other class I aaRS (Fig. 1). It is with two domains based on function and topology. The N-terminal catalytic domain is directly involved in tRNA acceptor stem binding, Tyr binding, and charging of amino acid to tRNA. The Tyr binding site is located in a deep and narrow pocket. Comparison of the *M. jannaschii* TyrRS and OMeTyrRS reveals the structural basis whereby the active-site mutations specifically accommodate OMeTyr and disfavor binding of Tyr. These include eliminating bulky residues to avoid stereochemical clashes against the larger substrate, as well as disruption of hydrogen bonds to the hydroxyl group of Tyr. Although four residues in the Tyr binding pocket were mutated to change specificity, no large backbone movements were observed. Even the overall shape of the binding pocket was not significantly changed upon mutation [13]. According to the above, by designing a series of aaRS mutating in the Tyr binding site of *M. jannaschii* TyrRS, with subsequently structure predictions and molecular dockings, one may obtain candidates specific for a desired unnatural amino acid. This is the origin of our idea presented in this paper.

In our study, we attempted to design aaRS being able to incorporate *p*-acetyl-L-phenylalanine (pAcPhe) into proteins, using Molecular Operating Environment (MOE) as the main software. The modules and parameters of MOE were optimized and verified to be suitable for *M. jannaschii* TyrRS mutants' structure predictions. Then, 60 aaRS were designed based on the conformation of *M. jannaschii* TyrRS. All of their predicted structures were refined and subjected to the model evaluations. After that, binding of these aaRS mutants with pAcPhe was evaluated by molecular docking. The mutants able to bind pAcPhe with reasonable poses were further subjected to energy minimization, and their binding affinities with pAcPhe were computed. Two mutants were chosen for their considerable binding affinities. They seemed to be able to aminoacylate the orthogonal tRNA with pAcPhe and incorporate pAcPhe into proteins in *E. coli* promisingly. They are currently under experimental verification. We reveal our results herein so that other researchers may improve this method and utilize it to develop new aaRS specific for other unnatural amino acids.

Material and methods

Validation of docking/scoring method. Performance of different docking/scoring methods may vary among different systems [14–17]. Therefore, the first step of our work was to test the docking/scoring methods of MOE/Dock to find out the optimal scheme suitable for *M. jannaschii* TyrRS mutants' structure predictions.

The MOE/Dock application is divided into a number of stages. Multiple methods are available for each stage. For the purpose of

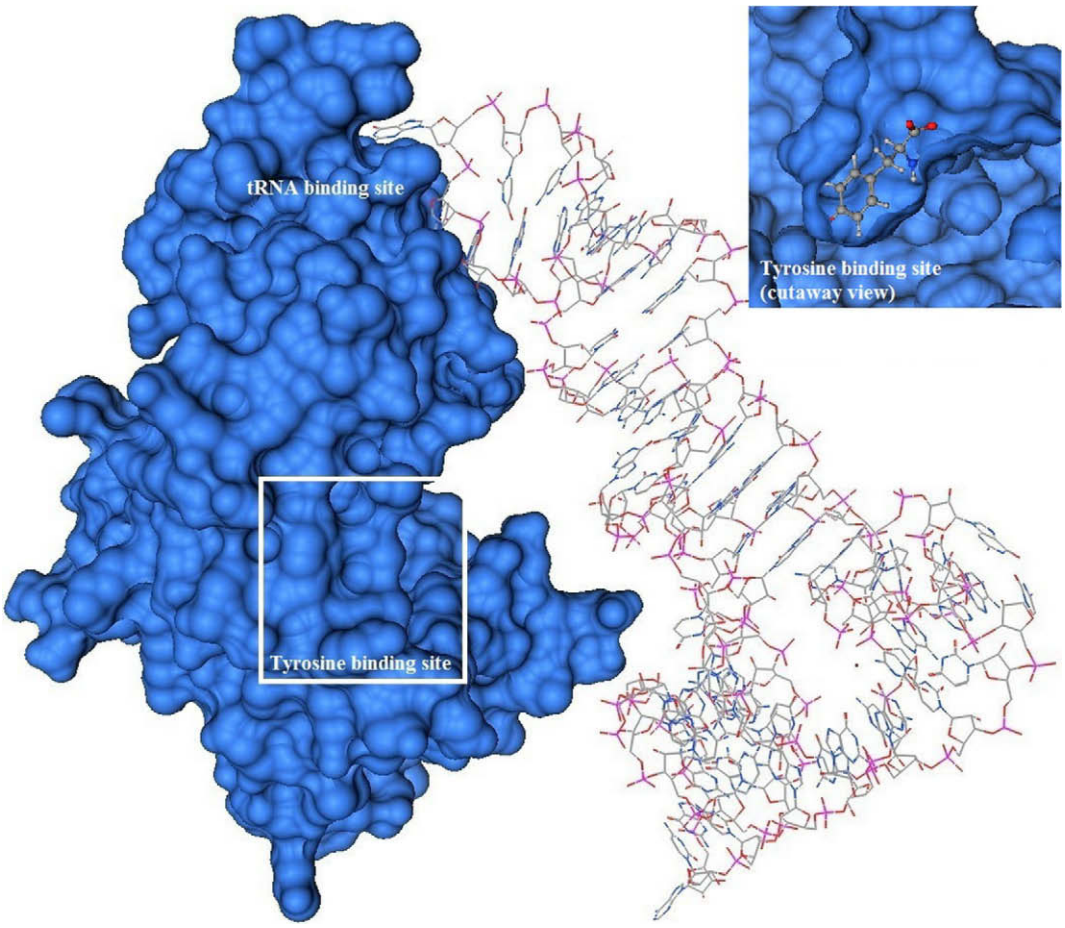

Fig. 1. The crystal structure of *Methanococcus jannaschii* tyrosyl-tRNA synthetase with tRNA (PDB ID: 1J1U) and cutaway view of the tyrosine binding site. The surface of tyrosyl-tRNA synthetase is colored blue. TRNA is rendered in the stick model and tyrosine in the ball and stick model with the carbon atoms colored gray, oxygen atoms red, nitrogen atoms blue and phosphorus atoms purplish red. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

validation, Tyr was docked into *M. jannaschii* TyrRS, using different combinations of methods. Definition of the docking site derived from the coordinate of Tyr in the crystal structure of *M. jannaschii* TyrRS complex (PDB ID: 1J1U). Only the scheme, with which root-mean-square-deviation (RMSD) value of the docked Tyr was closest to its coordinate in the crystal structure of *M. jannaschii* TyrRS, was considered optimal.

Homology modeling and refinement methods. In the homology modeling phase, the crystal structure of *M. jannaschii* TyrRS was utilized as template to build the TyrRS mutant specific for OMeTyr (OMeTyrRS) and design aaRS.

The rough models were refined with MOE/Minimization program using MMFF94x force field. MOE/Minimization calculates atomic coordinates that are local minima of a molecular energy function. All partial charges on the atoms of the rough models were calculated from the MMFF94x force field parameters. Then a three-step energy minimization was performed with gradual relaxation of restrictions for appropriate atoms. The non-hydrogen non-lone pair atoms were frozen during the first step, and the backbone atoms were frozen during the second step. In the last step, the backbone atoms were restricted with a force constant of 300, which was comparable to the strength of a bond in MOE. Energy minimization was set to be terminated when the root-mean-square-gradient fell below 0.1 for each step.

Validation of homology modeling and refinement methods. Not only docking/scoring method was employed as the essential tool in our study, but also homology modeling and refinement methods. To validate the homology modeling and refinement methods, the OMeTyrRS model quality was assessed by checking the geometric quality of backbone conformation and the statistics of non-bonded interactions between different atom types. PROCHECK [18–20] and ERRAT [21] were used. OMeTyr was also docked into the OMeTyrRS model to perform a docking analysis in addition.

Computation of binding affinity. The correct amino acid having the highest affinity with the active site of its corresponding aaRS takes precedence over other amino acids to integrate into the amino acid binding site [22]. Therefore, Generalized Born binding affinities [23] of designed aaRS and pAcPhe were calculated to find out the promising aaRS using MOE/LigX.

The three-dimensional structure of aaRS was fixed in our molecular docking studies described above. This simplification might be faulty since the structure of receptor should be flexible to certain extents when interacting with ligand. Thus an energy minimization of binding site in aaRS–ligand (amino acid) complex was performed before calculating binding affinity. Ligand and the residues within 8 Å of it were set to be flexible. After energy minimization, the binding affinity was calculated and reported in units of p*Ki* [23].

Hardware and software. The PROCHECK and ERRAT (http://nihserver.mbi.ucla.edu/SAVS/) validation were executed on-line. The other calculations involved were carried out by Molecular Operating Environment on a Windows workstation.

Results and discussions

Validation of docking/scoring method

Molecular docking was employed as the essential tool in our design. The following scheme was proved to be optimal for *M. jannaschii* TyrRS mutants' structure predictions. First, the Alpha Triangle method was used to generate the initiate conformations of ligand. All the conformations were subjected to scoring by London dG [24] method. Then, energy minimization of the ligand poses was performed considering receptor residues within 7 Å. The energy would be calculated using the Generalized Born solvation model [25], which was called force field method refinement. London dG rescoring was deployed at last. A maximal of 30 binding poses of ligand were retained in the outputting database, in descending order according to the final scores. If not specified above, parameters were set to their default values in MOE/Dock, including MMFF94x force field.

The results indicated that this docking/scoring scheme was able to successfully reproduce the coordinate of Tyr in *M. jannaschii* TyrRS. The RMSD value between the best-scored binding poses and Tyr in *M. jannaschii* TyrRS crystal structure was about 0.9 Å. It was also observed that only the binding poses with final scores more than S ($S = S_{\mathrm{Top}} - 0.5$, S_{Top} is the top score in the outputting database) could possibly reproduce the correct coordinate.

Binding mode and interactions of *M. jannaschii* TyrRS–Tyr (Fig. 2) indicated that, three hydrogen bonds were formed by the side chains of Tyr151, Gln155, Gln173 with amino group on Tyr; another two hydrogen bonds were formed by the side chains of Glu36 and Gln173 with carboxyl group on Tyr. Structural comparison of all class I aaRS reveals that, a set of residues are conserved among TyrRS from various species, corresponding to Tyr32, Tyr151, Gln155, Asp158, and Gln173 in *M. jannaschii* TyrRS [13]. It was reasonable to believe that, the five hydrogen bonds were important to TyrRS' catalysis of aminoacylating tRNA with Tyr, and so were they to the *M. jannaschii* TyrRS mutants'. Thus a criterion was proposed for the selection of correct poses in docking: the five hydrogen bonds between the designed aaRS and pAcPhe should be retained. This criterion was also used to design and evaluate aaRS mutants. Only the aaRS that retained the five hydrogen bonds with pAcPhe from molecular docking were considered possibly to be the promising candidates.

Validation of homology modeling and refinement methods

The OMeTyrRS model quality was assessed to validate the homology modeling and refinement methods.

The first assessment was carried out using Ramachandran's plot calculations computed with PROCHECK program by checking the detailed residue-by-residue stereochemical quality. Based on an analysis of 118 structures of resolution of at least 2.0 Å and R-factor no greater than 20%, a good quality model would be expected to have over 90% residues in the most favored regions [20]. The φ and ψ distributions of the non-glycine, non-proline residues are summarized in Fig. 3A. Altogether, 99.6% of their residues in OMeTyrRS model were in favored and allowed regions.

ERRAT, so-called "overall quality factor" for non-bonded atomic interactions, is a protein structure verification algorithm that is especially well-suited for evaluating the progress of crystallographic model building and refinement. The program works by analyzing the statistics of non-bonded interactions between different atom types. The normally accepted range is more than 50 for a high quality model [21]. In the current case shown in Fig. 3B, the ERRAT score for OMeTyrRS model is 99.3, within the range for a high quality model.

The ability to predict the correct ligand binding pose is important for a successful homology model. The docking analysis of OMeTyrRS model indicated that five hydrogen bonds were formed by the side chains of Tyr151, Gln155, Gln173 with amino group on OMeTyr, and the side chains of Glu36, Gln173 with carboxyl group on OMeTyr. It was consistent with the criterion mentioned in the end of part 3.1.

In brief, the geometric quality of the backbone conformation and non-bonded interactions between different atom types of OMeTyrRS model were all within the limits established for reliable structures. The docking study of this homology model was also in consistent with the conclusion from the study on interactions of

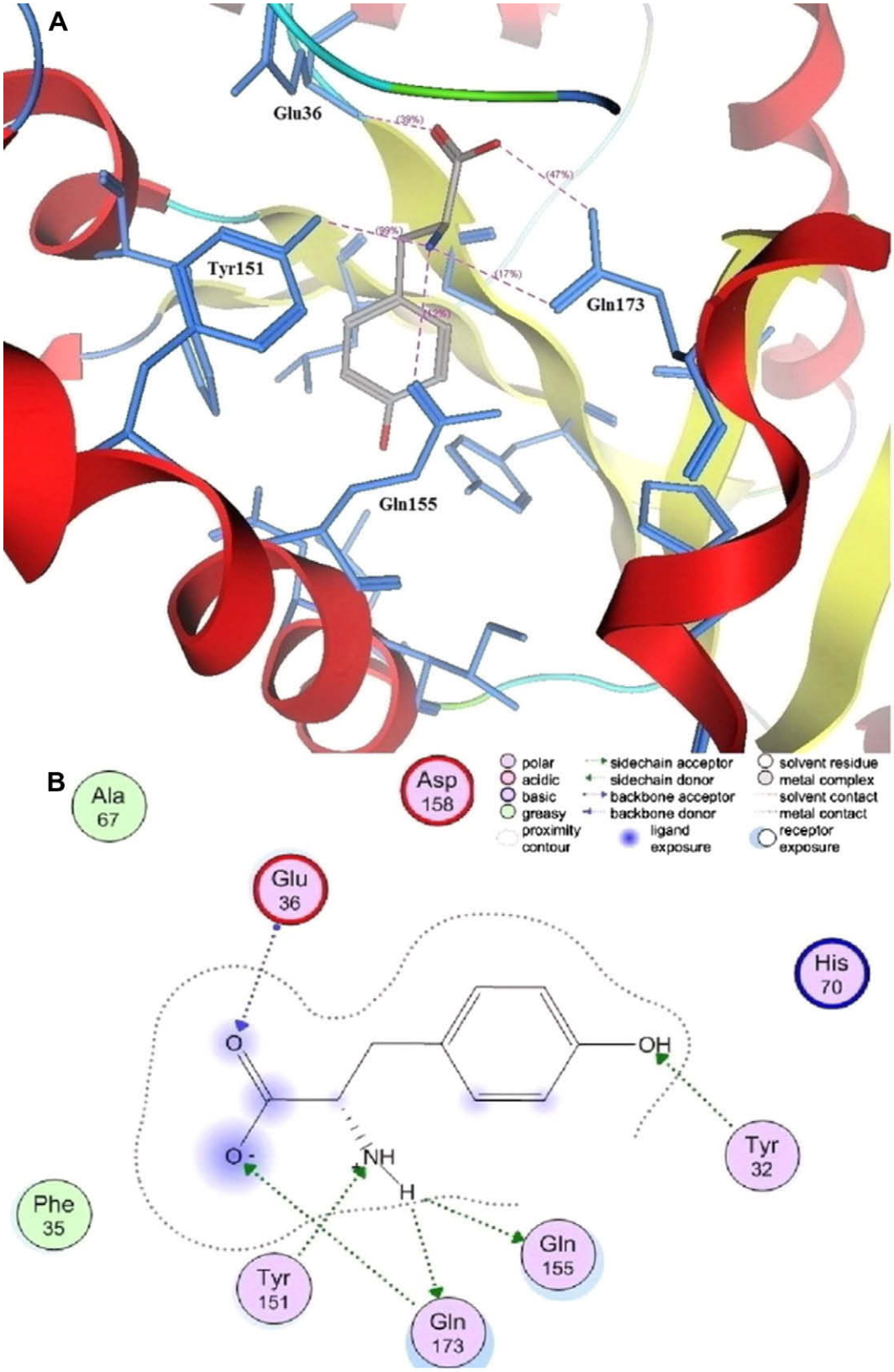

Fig. 2. (A) Binding mode of *Methanococcus jannaschii* tyrosyl-tRNA synthetase with tyrosine. Residues around tyrosine are rendered in blue stick models. The purple dashed lines indicate the hydrogen bonds. The percentage is the qualifying score of each hydrogen bond. (B) 2D view of interactions of *Methanococcus jannaschii* tyrosyl-tRNA synthetase and tyrosine. Hydrogen bonds are represented with green dashed lines. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

M. jannaschii TyrRS–Tyr and conserved residues among all class I TyrRS. All evidences suggested that a reasonable homology model for OMeTyrRS had been obtained, which proved homology modeling and refinement methods in the meanwhile.

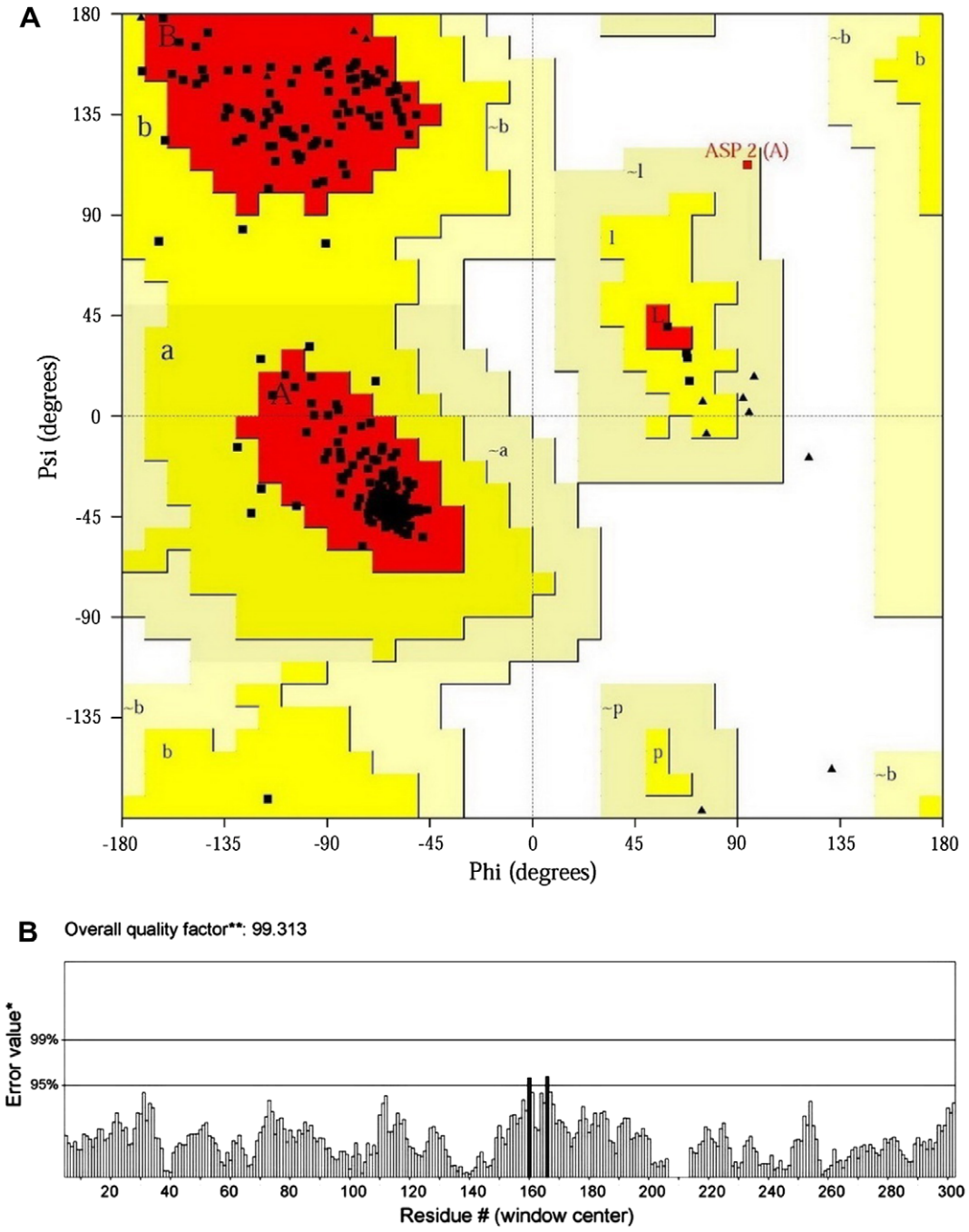

Fig. 3. (A) Ramachandran's map of OMeTyrRS model. (B) ERRAT histogram of OMeTyrRS model. *On the error axis, two lines are drawn to indicate the confidence with which it is possible to reject regions that exceed that error value.

To obtain an accurate model, it is of importance that appropriate steps are built into the process to assess the quality of the model [26]. The PROCHECK and ERRAT assessments were also deployed to qualify all designed aaRS models.

Molecular design and molecular docking evaluation

The primary goal was to design aaRS being able to bind with pAcPhe in the amino acid binding site with correct pose.

Important conclusions were drawn by comparing *M. jannaschii* TyrRS with OMeTyrRS: no large backbone movements were observed; even the overall shape of amino acid binding pocket was not significantly changed upon mutation; residues were mutated to smaller amino acids to avoid stereochemical clashes against the larger substrate [13]. Taking into account that pAcPhe would hold approximately the same location as Tyr in TyrRS for forming the five conserved hydrogen bonds, pAcPhe was superposed on Tyr in *M. jannaschii* TyrRS–Tyr complex (Fig. 4). Apparently Tyr32 and Asp158 were both involved in a steric clash with acetyl on pAcPhe, as well as Ile159. These residues should be mutated to adjust the pocket shape for pAcPhe binding.

Tyr32 was equivalent to amino acid with four-carbon-length (4C) side chain. It would be replaced by non-polar amino acid with shorter side chain in order to reduce steric clash and provide hydrophobic environment to acetyl on pAcPhe. Only Val, Leu, Ile, Gly and Ala might be the options; Asp158 was mutated to amino

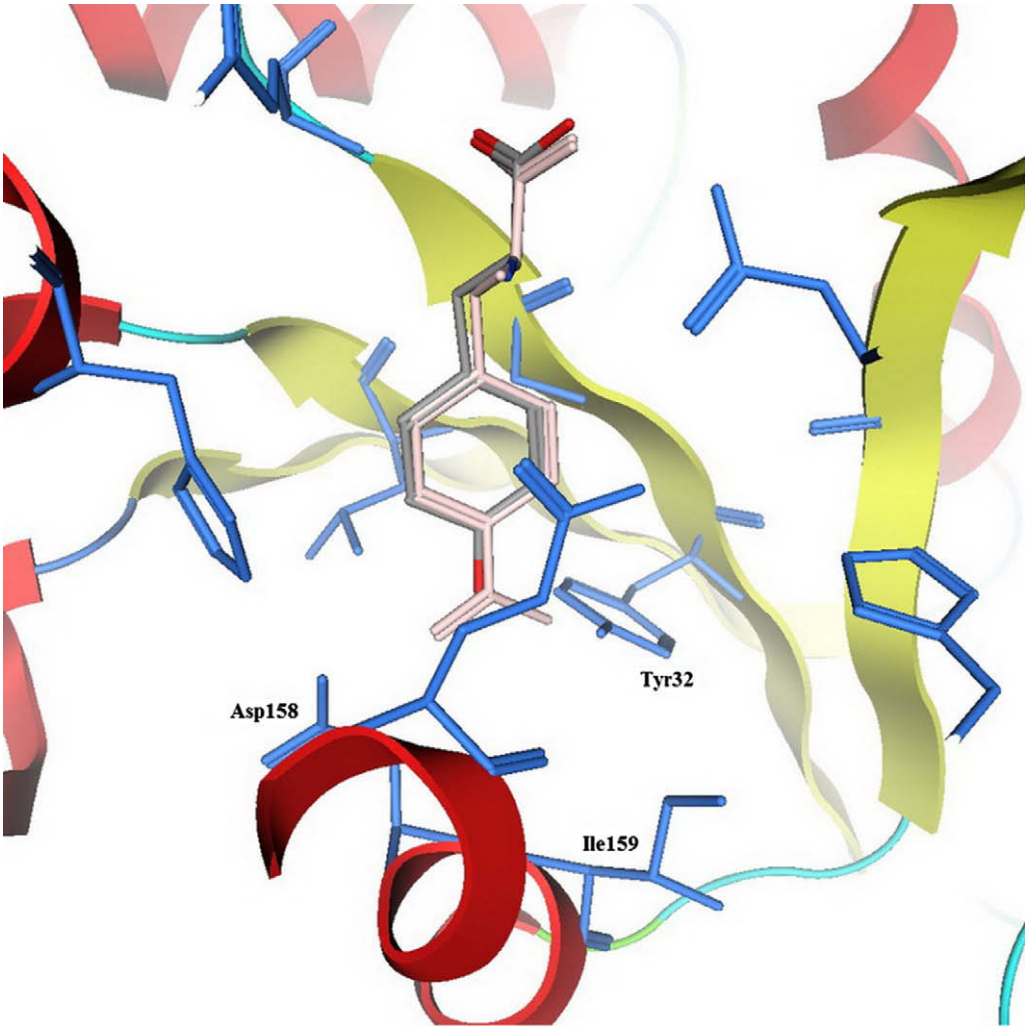

Fig. 4. *p*-Acetyl-L-phenylalanine in Tyr binding site of *Methanococcus jannaschii* tyrosyl-tRNA synthetase. Residues around tyrosine are rendered in blue stick models. *p*-Acetyl-L-phenylalanine superposing on tyrosine is rendered in pink stick models. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

acid with side chain shorter than 3C for the same reason. Considering that carbonyl was a potential hydrogen-bond receptor, Ser was also included, so Asp158 could be substituted by Gly, Ala or Ser; Ile159, which was mutated separately, was designed to be replaced by Val, Gly or Ala. So we designed the 60 aaRS. They were homology modeled using *M. jannaschii* TyrRS as template. All of them were refined and their predicted structures were proved. Ramachandran's plot calculations for the 60 aaRS models showed the percentage of residues belonging to the most favored regions was above 94%. The ERRAT scores were all above 99.000.

Bindings of these designed aaRS with pAcPhe were evaluated by molecular docking. Only 15 of them turned out to be able to form five hydrogen bonds with pAcPhe. They were the promising candidates for further evaluation in the next stage.

Further evaluation through binding affinity computations

Binding affinity computations (Table 1) showed that X47, X02 and X17, which shared the same character, had considerable binding affinities with pAcPhe. It suggested the desired docking of aaRS–pAcPhe was benefit from mutations of Tyr32Leu and Asp158Gly. The reason might be that in *M. jannaschii* TyrRS Tyr32 and Asp158 were both directly involved in hydrogen-bond interactions with the hydroxyl of Tyr. Mutations of these two residues resulted in a loss of them. Tyr32's mutating to smaller amino acid Leu not only released a steric clash of active-site residues against the methyl group of pAcPhe, but also provided the hydrophobic environment. Asp158 was also close to the acetyl side chain. When this residue was mutated to Gly, it reduced the steric effect, leaving space for the additional acetyl group of pAcPhe.

The mutations of Tyr32Leu and Asp158Gly were found consistent with the LW1 and LW5 reported by experimental screening, which were able to incorporate pAcPhe [27]. The designed aaRS with this feature could bind pAcPhe with predicted binding affinities of 10.716, 10.711 and 10.641, and all of them were on the top of Table 1. X47 and X02 were computed to have binding affinities comparable with LW1 and LW5 (Table 1). They seemed to be able to aminoacylate the orthogonal tRNA with pAcPhe and incorporate pAcPhe into proteins in *E. coli* promisingly, which fulfilled our original goal of this study.

Conclusions

We have designed some aaRS through a combination of homology modeling, molecular docking and binding affinity computation with the purpose of incorporating pAcPhe into proteins in *E. coli*. Structural comparison of *M. jannaschii* TyrRS, OMeTyrRS and all class I aaRS indicated that Tyr32, Asp158 and Ile159 should be

Table 1
Computed binding affinities of designed aminoacyl-tRNA synthetase with *p*-acetyl-L-phenylalanine.

ID	Tyr32	Asp158	Ile159	Binding affinity (p*Ki*)
wtTyrRS*	Tyr	Asp	Ile	11.688
LW1**	Leu	Gly	Cys	10.767
LW5**	Leu	Gly	Thr	10.727
X47	Leu	Gly	Val	10.716
X02	Leu	Gly	Ile	10.711
X17	Leu	Gly	Gly	10.641
X51	Ile	Ser	Val	10.613
X05	Ile	Gly	Ile	10.547
X08	Val	Gly	Ile	10.533
X53	Val	Gly	Val	10.529
X31	Leu	Ala	Ala	10.520
LW6**	Ala	Gly	Gly	10.427
X20	Ile	Gly	Gly	10.406
X44	Ala	Gly	Ala	10.404
X14	Ala	Gly	Ile	10.348
X43	Ala	Ala	Ala	10.329
X29	Ala	Gly	Gly	10.302
X26	Gly	Gly	Gly	10.243
X46	Leu	Ala	Val	10.211

* *Methanococcus jannaschii* tyrosyl-tRNA synthetase was docked with tyrosine, and the others, with *p*-acetyl-phenylalanine.
** Leu162 and Ala167 were also mutated in LW1, LW5, LW6, but not listed.

substituted by relatively small amino acids to reduce steric clash between *M. jannaschii* TyrRS and acetyl on pAcPhe. 60 aaRS were generated by combination of the three residues' mutations. The structures of the 60 aaRS were modeled using the conformation of *M. jannaschii* TyrRS as template. Only 15 aaRS retained the five hydrogen bonds with pAcPhe from molecular docking outputs and they were considered possibly to be promising. In particular, X47 and X02 were computed to have binding affinities comparable to LW1 and LW5, which were reported to be able to incorporate pAcPhe by experimental screening. X47, X02, LW1 and LW5 shared the same character, the mutations of Tyr32Leu and Asp158Gly. So X47 and X02 seemed to be able to aminoacylate the orthogonal tRNA with pAcPhe and incorporate pAcPhe into proteins in *E. coli* promisingly. They are currently under experimental verification in our laboratory.

The results show that the combination of homology modeling and molecular docking is a feasible method to filter inappropriate mutations in molecular design and point out beneficial mutations. This new strategy may be able to accelerate the efficiency of screening unlimited numbers of protein mutants. We reveal our results so that other researchers may improve this method and utilize it to develop new aaRS specific for other unnatural amino acids or design molecule with a specific function.

Acknowledgments

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