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Expanding the Genetic Code of *Escherichia coli* with Phosphotyrosine

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Abstract

Protein phosphorylation is one of the most important post-translational modifications in nature. However, the site-specific incorporation of *O*-phosphotyrosine into proteins *in vivo* has not yet been reported. Endogenous phosphatases present in cells can dephosphorylate phosphotyrosine as a free amino acid or as a protein residue. Therefore, we deleted the genes of five phosphatases from the genome of *Escherichia coli* with the aim of stabilizing phosphotyrosine. Together with an engineered aminoacyl-tRNA synthetase (derived from *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase) and an elongation factor Tu variant, we were able to co-translationally incorporate *O*-phosphotyrosine into the super-folder green fluorescent protein at a desired position *in vivo*. This system will facilitate future studies of tyrosine phosphorylation.

Keywords

phosphotyrosine; genetic code expansion; phosphatase; tyrosine phosphorylation; aminoacyl-tRNA synthetase; elongation factor

Phosphorylation of tyrosine residues in proteins is a crucial post-translational modification that plays key roles in regulating many prokaryotic and eukaryotic processes including growth control, cell cycle control, differentiation, cell shape and movement, gene transcription, synaptic transmission, and bacterial virulence [1–4]. The human genome contains at least 90 protein tyrosine kinases (PTKs) which phosphorylate tyrosine residues in proteins and 107 protein tyrosine phosphatases (PTPs) that can remove the phosphate from *O*-phospho-tyrosine (pTyr) in proteins, opposing the functions of PTKs [5,6]. The balanced actions of PTKs and PTPs regulate the phosphorylation of tyrosine residues, and

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Author Contributions

CF and DS conceived and designed experiments, and wrote the paper. CF and KI carried out experiments. CF and DS discussed and analyzed data.

abnormalities result in the pathogenesis of numerous inherited or acquired human diseases from cancer to immune deficiencies [1].

Due to the importance of tyrosine phosphorylation, many strategies have been adopted to synthesize peptides and proteins containing purely phosphorylated tyrosine residues. Using the method of *in vitro* translation which involves read-through of an amber (UAG) stop codon by a chemically misacylated suppressor tRNA, pTyr was incorporated into proteins at controlled positions [7]. Later, caged pTyr was chemically synthesized to overcome the low incorporation efficiency caused by the poor binding of the elongation factor Tu (EF-Tu) with charged phosphate group of pTyr-tRNA; subsequently the caging group could be removed by light [8]. Recently, several pTyr analogs were genetically incorporated into proteins *in vivo* [9,10]. Sulfotyrosine was incorporated into proteins for the study of tyrosine sulfation [9]. Another *in vivo* incorporated pTyr mimic is *p* carboxylmethy-phenylalanine which was previously used as a replacement for pTyr in SH2 domain binding [10,11]. Moreover, *p* (phosphonoamino)-phenylalanine was incorporated into proteins by chemically modifying a genetically installed *p* azidophenyalanine residue *in vitro* [12]. However, the direct incorporation of pTyr into proteins *in vivo* has not been reported yet. Here, we described an approach with integrated strategies to genetically incorporate pTyr into proteins in *E. coli* cells.

Materials and methods

General molecular biology and plasmid constructions

Oligonucleotide synthesis, DNA sequencing and mass spectrometry were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. The chemicals in this study were purchased from Sigma-Aldrich or ChemImpex. *E. coli* TOP10 cells (Life Technologies) were used for general cloning. Plasmids: The genes of *M. jannaschii* tyrosyl-tRNA synthetase (*mj*TyrRS), $tRNA^{Tyr}$, and EF-Tu were cloned by PCR from laboratory inventory and inserted into the *pTech* plasmid. The synthetase and EF-Tu genes were placed under the constitutive *lpp* promoter and the tRNA gene was placed under constitutive *proK* promoter. The gene of codon optimized super-folder green fluorescent protein (sfGFP) with C terminal His_6 tags was cloned into the *pBAD* plasmid and placed under the control of the inducible arabinose promoter. All the cloning experiments were performed by using the Gibson Assembly kit (New England Biolabs). The mutations in protein genes were made by the QuikChange II mutagenesis kit (Agilent Life Sciences).

Phosphatase activity assay

The phosphatase activity was determined by the Tyrosine Phosphatase Assay System (Promega) measuring the absorbance of a molybdate:malachite green:phosphate complex following the manufacturer's instructions. The reactions were read in the BioTeK micro-plate reader.

Protein expression and purification

The expression strains were grown in 500 ml of LB media supplemented with 0.1 mg/ml ampicillin at 37°C to an absorbance of 0.6 0.8 at 600 nm, then protein expression was

induced by the addition of 1 mM isopropyl β D-thiogalactopyranoside (IPTG) or 10 mM arabinose. Cells were incubated at 25°C for an additional 12 h and harvested by centrifugation at 5000 × g for 10 min at 4 °C. The cell paste was suspended in 15 ml of lysis buffer (50 mM Tris pH 8, 300 mM NaCl, 20 mM imidazole) and broken by sonication. The crude extract was centrifuged at 20,000 × g for 30 min at 4°C. The soluble fraction was loaded onto a column containing 1 ml of Ni-NTA resin (Qiagen) previously equilibrated with 20 ml of lysis buffer. The column was washed with 20 ml of lysis buffer, and then eluted with 2 ml of 50 mM Tris (pH 7.5), 300 mM NaCl, 200 mM imidazole. The purified protein was dialyzed with 50 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1mM DTT and 50% glycerol, and stored at −80°C for further studies.

tRNA transcription, purification, and ^{32}P labeling

Template plasmids containing tRNA genes were purified with the plasmid maxi kit (Qiagen), and 100 μg of plasmid was digested with BstNI (New England Biolabs). The BstNI digested template DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation and dissolved in double distilled water. The transcription reaction (40 mM Tris pH 8; 4 mM each of UTP, CTP, GTP, and ATP at pH 7.0; 22 mM $MgCl_2$; 2 mM spermidine; 10 mM DTT; 6 μg pyrophosphatase (Roche Applied Science); 60 μg/mL BstNI digested DNA template, approximately 0.2 mg/ml T7 RNA polymerase) was performed in 10 ml reaction volumes for overnight at 37°C. The tRNA was purified on a 12% denaturing polyacrylamide gel containing 8 M urea and TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA). UV shadowing identified the pure tRNA band, which was excised and extracted three times with 1M sodium acetate pH 5.3 at 4°C. The tRNA extractions were then ethanol precipitated, dissolved in RNase-free distilled water, pooled, and finally desalted using a Biospin 30 column (BioRad). The tRNA was refolded by heating to 100°C for 5 min and slow cooling to room temperature. At 65°C, $MgCl_2$ was added to a final concentration of 10 mM to aid folding. 16 μM folded tRNA in 50 mM Tris (pH 8.0), 20 mM $MgCl_2$, 5 mM DTT and 50 μM NaPPi was incubated at room temperature for 1 hr with approximately 0.2 mg/ml CCA-adding enzyme and 1.6 μCi/μl of [α-^{32}P]-labeled ATP (PerkinElmer). The sample was phenol/chloroform extracted and then passed over a Bio-spin 30 column (Bio-Rad) to remove excess ATP.

Aminoacylation assay

The assay was modified from the original method [13]. A 20 μl reaction contained the following components: 50 mM HEPES-KOH (pH 7.2), 25 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, 25 μg/ml pyrophosphatase (Roche Applied Science), 1 μM tRNA. All tRNA aminoacylation levels were determined at 37°C with synthetase, 10 nM ^{32}P-labeled tRNA. Time points were taken at 5 min, 10 min and 30 min by removing 2 μl aliquots from the reaction and immediately quenching the reaction into an ice-cold 3 μl quench solution (0.66 μg/μl nuclease P1 (Sigma) in 100 mM sodium citrate (pH 5.0)). For each reaction, 2 μl of blank reaction mixture (containing no enzyme) was added to the quench solution as the start time point. The nuclease P1 mixture was then incubated a room temperature for 30 min and 1 μl aliquots were spotted on PEI-cellulose plates (Merck) and developed in running buffer containing 5% acetic acid and 100 mM ammonium acetate. Radioactive spots of AMP and AA-AMP (representing free tRNA and aminoacyl-tRNA, respectively) were separated

and then visualized and quantified by phosphorimaging in a Molecular Dynamics Storm 860 phosphorimager (Amersham Biosciences). The ratio of amino-acyl-tRNA to total tRNA was determined to monitor reaction progress.

Mass spectrometry analyses

The purified proteins were trypsin digested by a standard in-gel digestion protocol, and analyzed by LC-MS/MS on an LTQ Orbitrap XL (Thermo Scientific) equipped with a nanoACQUITY UPLC system (Waters). A Symmetry C18 trap column (180 μm×20 mm; Waters) and a nanoACQUITY UPLC column (1.7 μm, 100 μm×250 mm, 35°C) were used for peptide separation. Trapping was done at 15 μL min^{-1}, 99% buffer A (water with formic acid (0.1 %)) for 1 min. Peptide separation was performed at 300 nL min^{-1} with buffer A and buffer B (CH_3CN containing 0.1% formic acid). The linear gradient (51 min) was from 5% buffer B to 50% B at 50 min, to 85% B at 51 min. MS data were acquired in the Orbitrap with one microscan, and a maximum inject time of 900 ms followed by data-dependent MS/MS acquisitions in the ion trap (through collision induced dissociation, CID). The Mascot search algorithm was used to search for the appropriate noncanonical substitution (Matrix Science, Boston, MA).

Results

Deleting phosphatases for phosphotyrosine from *E. coli*

One challenge for *in vivo* incorporation of pTyr into proteins is the stability of pTyr in living cells which have many endogenous phosphatases. These enzymes may dephosphorylate pTyr either as free amino acid, or as a constituent of proteins. In *E. coli* there are about 90 phosphatases according to gene ontology [14], some of them may be specific for pTyr. Therefore, we wanted to decrease the phosphatase activity for pTyr in *E. coli* by deleting the genes of relevant phosphatases. As detailed biochemical knowledge on pTyr phosphatases does not exist, we selected 14 possible ones including SerB, SurE, PgpA, PgpB, PgpC, PphA, PphB, NudJ, HisB, YedJ, YedP, AphA, PhoA, and YnbD. We utilized the ASKA strain collections which overexpress individual gene of *E. coli* [15], and measured the phosphatase activities in the crude extracts of the corresponding strains for selected genes. The results showed that five phosphatases had obviously higher activities for pTyr than other phosphatase candidates; these are SerB, the phosphoserine phosphatase; PgpA, a phosphatidyl-glycerophosphatase in membranes; PphB, a phosphoprotein phosphatase involved in the misfolded protein stress; PhoA, a nonspecific alkaline phosphatase; and AphA, a nonspecific acidic phosphatase (Fig. 1). Then we knocked out the genes of these five phosphatases from the genome of *E. coli* TOP10 by recombination [16] to form the Δ5P strain, which grew at with a doubling time of 28 ± 4 min (12% slower than the wild-type parent) in LB medium. The phosphatase activity for pTyr in the crude extract of Δ5P cells was only about 30% of that in wild-type TOP10 cells.

Screening tyrosyl-tRNA synthetase (TyrRS) variants for pTyr-tRNA formation

Due to the poor cellular uptake of pTyr, the instability of pTyr in cells, as well as the need of evolving multiple translational components (TyrRS and EF-Tu) for pTyr incorporation, traditional strategies in genetic code expansion cannot be used to select TyrRS variants

specific for pTyr efficiently [17]. To address this issue, we performed an *in vitro* screening approach based on the TyrRS variant activities of charging the tRNA with pTyr. Previously, an orthogonal pair of *M. jannaschii* TyrRS (*mj*TyrRS) and $tRNA^{Tyr}_{CUA}$ was described [9] to incorporate sulfotyrosine into proteins, an amino acid structurally similar to pTyr. Thus, we started with this variant (Y32L, L65P, D158G, I159C, L162K) for further engineering. Based on the crystal structures of *mj*TyrRS and its variants [18–20], we chose four residues (positions of 32, 65, 108, and 109) which possibly contact for the additional negatively charged oxygen atom (Fig. 2A), to be replaced with all other 19 amino acids at each position. We also included the amino acid substitution of D286R in each variant which was shown to increase $tRNA^{Tyr}_{CUA}$ anticodon recognition by the *mj*TyrRS [18]. In total 76 variants were made. Each variant was individually overexpressed, purified, and tested for aminoacylation activity with pTyr. Finally, we obtained two variants with the highest activities: pYRS1 (Y32L, L65R, D158G, I159C, L162K, D286R) and pYRS2 (Y32L, L65K, D158G, I159C, L162K, D286R) (Fig. 2B). The positively charged arginine and lysine residues at position 65 may facilitate the binding of negatively charged pTyr.

Screening EF-Tu variants for pTyr-tRNA binding

It is known that the EF-Tu binds tRNAs bearing negatively charged amino acids poorly [21]. Previously, we engineered EF-Tu to efficiently facilitate incorporation of another phosphoamino acid, phosphoserine, into proteins [22–24]. Phosphotyrosine also has two negative charges, so we rationally designed EF-Tu variants for better binding with pTyr to increase its incorporation into proteins. Based on the co-crystal structure of EF-Tu with Phe-$tRNA^{Phe}$ (Fig. 3A), we introduced the amino acid substitutions of E216V and D217G from the EF-Tu variant which could facilitate phosphoserine incorporation [24]. These two substitutions of negatively charged residues (Asp and Glu) facilitate the binding of EF-Tu with negatively charged amino acids. As for pTyr, it needs more binding space than phosphoserine due to its rigid aromatic ring, thus we replaced the residue at position 219, which may contact with the phosphate group in pTyr, with the other 19 amino acids and utilized the well-established super-folder green fluorescent protein (sfGFP) readthrough assay [25] to test the residue substitution effects on pTyr incorporation. The Δ5P strain constructed above was used as the host, and the orthogonal pair of engineered pYRS1 and Nap1 $tRNA^{Tyr}$ which was shown to comprehensively increase non-canonical amino acid incorporation by the *mj*YRS system [26] was co-expressed with each EF-Tu variant, individually. By using the sfGFP bearing an amber stop codon at a permissive position 143 as the reporter [27], we quantitated the readthrough of the amber stop codon from fluorescence readings. The results showed that the replacement of position 219 with amino acids with smaller side chains (Ala, Gly, and Ser) could increase the pTyr incorporation (Fig. 3B). Here, we selected the best variant (E216V, D217G, F219G) as EF-pY in later experiments.

Incorporation of pTyr into protein in vivo

First we used the sfGFP readthrough assay to evaluate the essentiality of each component mentioned above in pTyr incorporation (Fig. 4A). The results showed that the phosphatase knockout strain, the engineered TyrRS and EF-Tu variants were equally important. Without any one of the Δ5P strain, the pYRS variant, or the EF-pY variant, the fluorescence reading

dropped to the background level (no pTyr in the media). Then we purified the sfGFP bearing an amber stop codon at position 143 which was expressed with all these components (Fig. 4B). The yield of pTyr-containing sfGFP was about 20 mg/L in LB media. The mass spectrometric analysis confirmed pTyr incorporation at the designed position 143 (Fig. 5).

Discussion

Compared with the expression of wild-type sfGFP under the same growth condition (Fig. 4B), the yield of pTyr-containing sfGFP was about 5%. The major amino acid incorporated at position 143 is pTyr, because we showed that if we did not provide pTyr in the growth media, the readthrough of the stop codon in sfGFP is only 20% of that with pTyr in the media (Fig. 4A). We also detected the incorporation of glutamine and lysine at this position, which was probably from the near cognate suppression of the amber stop codon [28]. Although the yield of pTyr-containing protein was relatively low, our work forms a solid step toward highly efficient incorporation of pTyr into proteins *in vivo*. Below are some approaches that could increase pTyr incorporation.

In this work, we used a high concentration of pTyr in the growth media to overcome the poor cellular uptake of pTyr [29]. However, it is necessary to look for proper transporters for pTyr which may be from other organisms. A similar strategy was successful in making a semi-synthetic *E. coli* with an expanded genetic alphabet by introducing an algal nucleotide triphosphate transporter for unnatural nucleoside triphosphates uptake [30].

Although we removed five phosphatase genes from the *E. coli* genome, there remained 30% pTyr phosphatase activity in the cells. We did detect by mass spectrometry analysis Tyr incorporation at position 143 in sfGFP; this may be due to dephosphorylation by unknown protein phosphatases. Thus, inactivating additional phosphatases or adding phosphatase inhibitors may help to increase pTyr incorporation. For this purpose, advanced techniques such as multiplex automated genome engineering (MAGE) can be utilized to inactivate multiple phosphatases simultaneously [31]. However, deleting more phosphatases may affect cell growth due to their important roles in cell physiology. And indeed, our five-phosphatase knockout strain grew relatively poorly in M9 minimal media.

In this study, we used rational design to engineer TyrRS and EF-Tu variants for pTyr. The advantage of advanced protein evolution systems such as MAGE and phage-assisted continuous evolution (PACE) [32,33] in creating larger libraries of variants and selecting variants automatically will help us to find more efficient and specific AARS and EF-Tu variants for pTyr incorporation.

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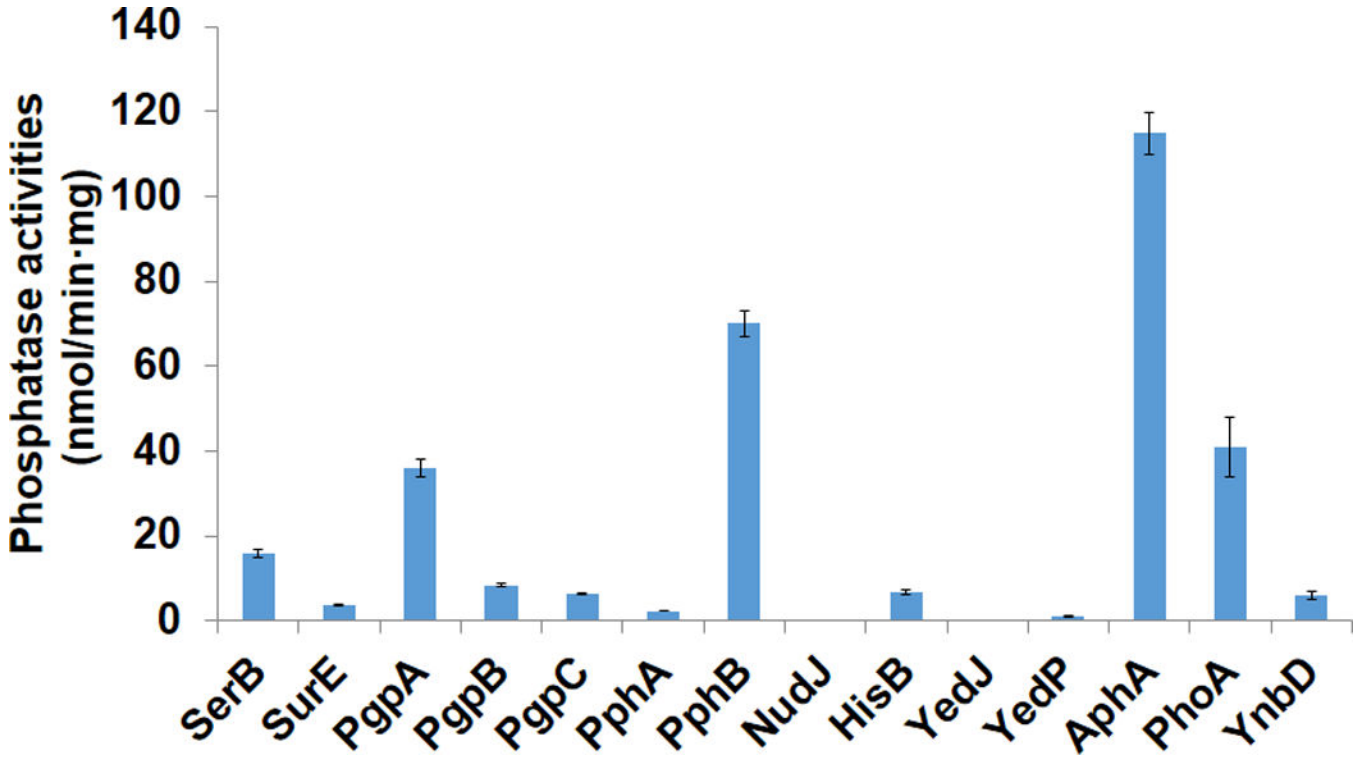

Fig. 1.

Activities of selected phosphatases for phosphotyrosine. The assays were performed according to the manufacturer's instructions. 10 μL crude extracts were added to the reaction mixtures for measurement. The background value for wild-type TOP10 cells was set as zero, and the mean values and standard errors were calculated from three replicates.

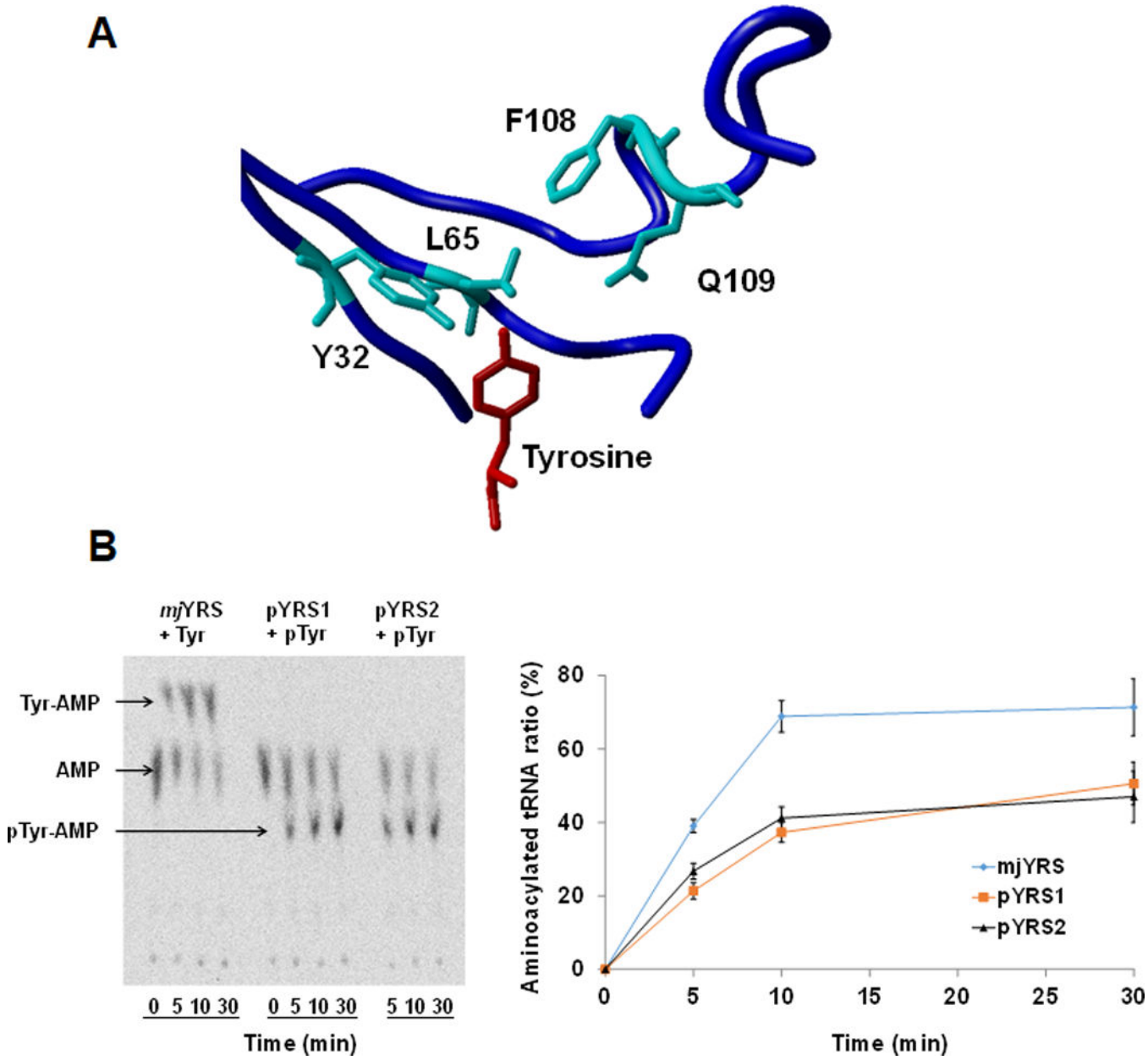

Fig. 2.

(A) The crystal structure of tyrosine binding site of the wild-type *mj*YRS (based on Protein Data Bank structure 1J1U). (B) The aminoacylation assay [13] of wild-type *mj*YRS for tyrosine and its variants for pTyr. 10 μM wild-type *mj*TyrRS or 100 μM *mj*TyrRS variants and 1 mM tyrosine or 10 mM pTyr were used in the assay. The mean values and standard errors were calculated from three replicates.

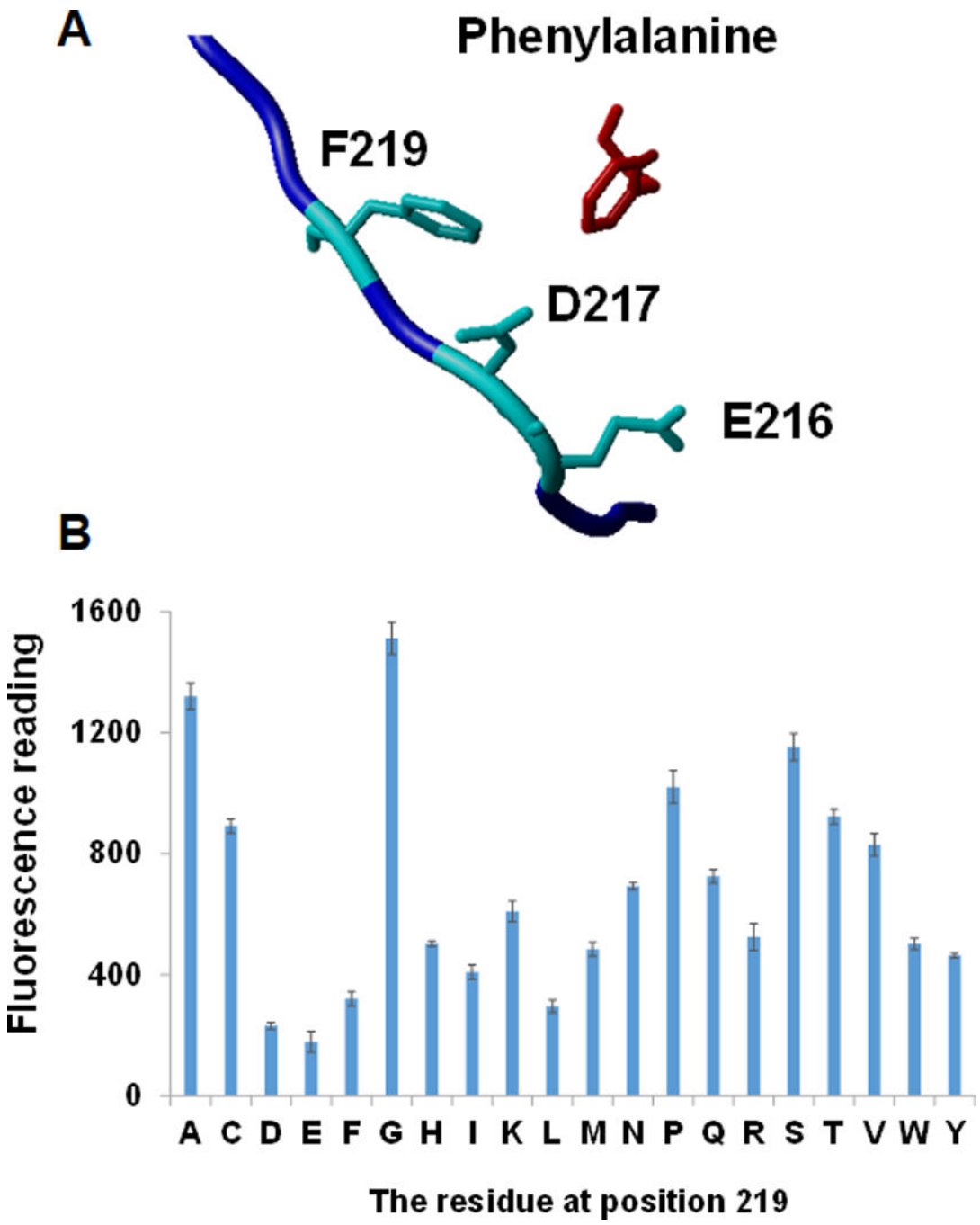

Fig. 3.

(A) The model of the amino acid binding pocket of *E. coli* EF-Tu with phenylalanine charged tRNA (based on Protein Data Bank structure 1OB2). (B) The sfGFP readthrough assay for EF-Tu variants. The background fluorescence for wild-type TOP10 cells was set as zero. The mean values and standard errors were calculated from three replicates.

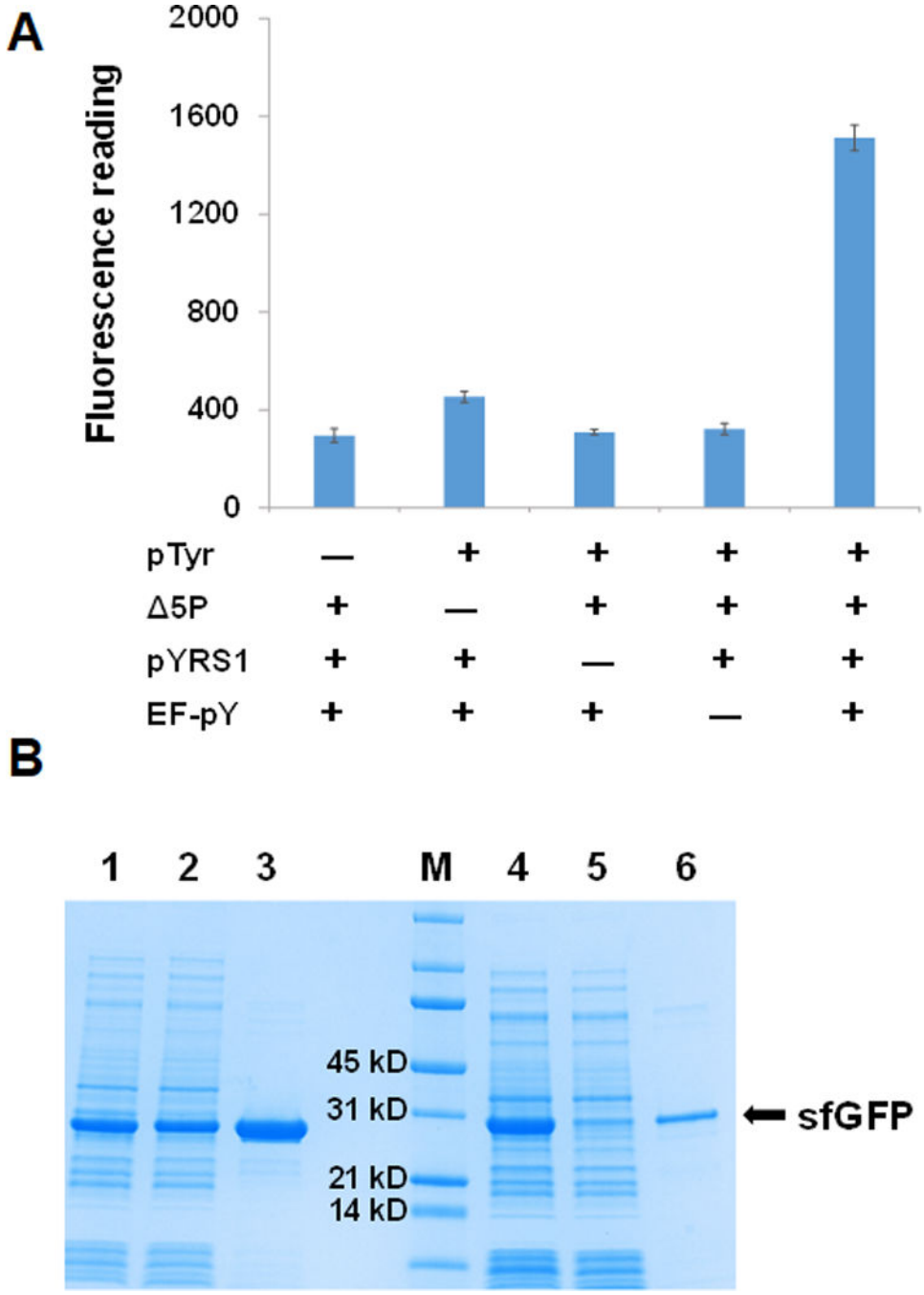

Fig. 4.

(A) The effects of components in pTyr incorporation. The background fluorescence for wild-type TOP10 cells was set as zero. 10 mM pTyr was used in the growth media. The mean values and standard errors were calculated from three replicates. (B) The purification of pTyr-containing and wild-type sfGFP. Lane 1, 2, and 3 are the crude extract, the soluble fraction, and the elution from the cells expressing the wild-type sfGFP, individually. Lane 4, 5, and 6 are the crude extract, the soluble fraction, and the elution from the cells expressing

pTyr-containing sfGFP, separately. The same volumes of fractions were loaded on the SDS-PAGE gel.

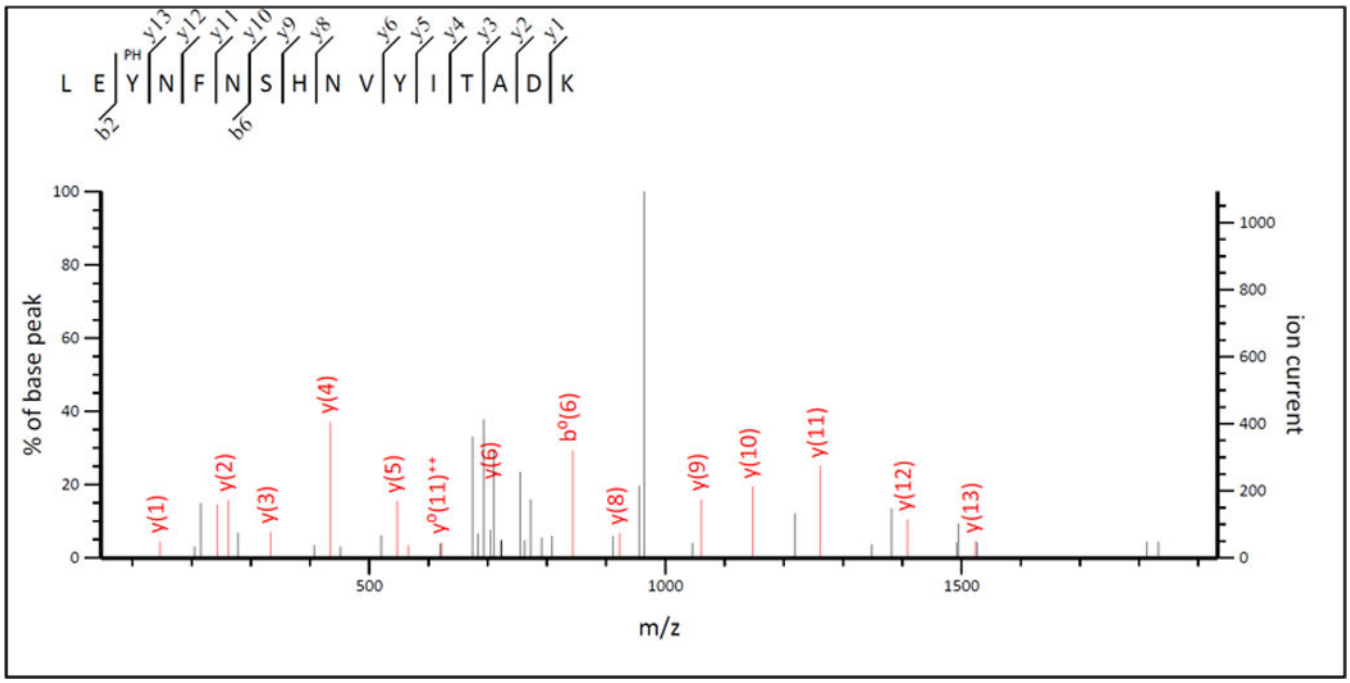

Fig. 5.

The tandem mass spectrum of the peptide (residues 141–156) LEY^{PH}NFNSHNVYITADK (ion score 57) from purified sfGFP with one amber codon at position 143. Y^{PH} denotes pTyr incorporation. The partial sequences of the peptides containing the pTyr can be read from the annotated b or y ion series.