

Human tryptophanyl-tRNA synthetase is switched to a tRNA-dependent mode for tryptophan activation by mutations at V85 and I311

Li-Tao Guo¹, Xiang-Long Chen¹, Bo-Tao Zhao¹, Yi Shi¹, Wei Li¹, Hong Xue² and You-Xin Jin^{1,*}

¹State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031 and ²Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

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ABSTRACT

For most aminoacyl-tRNA synthetases (aaRS), their cognate tRNA is not obligatory to catalyze amino acid activation, with the exception of four class I (aaRS): arginyl-tRNA synthetase, glutamyl-tRNA synthetase, glutaminyl-tRNA synthetase and class I lysyl-tRNA synthetase. Furthermore, for arginyl-, glutamyl- and glutaminyl-tRNA synthetase, the integrated 3' end of the tRNA is necessary to activate the ATP-PPi exchange reaction. Tryptophanyl-tRNA synthetase is a class I aaRS that catalyzes tryptophan activation in the absence of its cognate tRNA. Here we describe mutations located at the appended $\beta 1$ - $\beta 2$ hairpin and the AIDQ sequence of human tryptophanyl-tRNA synthetase that switch this enzyme to a tRNA-dependent mode in the tryptophan activation step. For some mutant enzymes, ATP-PPi exchange activity was completely lacking in the absence of tRNA^{Trp}, which could be partially rescued by adding tRNA^{Trp}, even if it had been oxidized by sodium periodate. Therefore, these mutant enzymes have strong similarity to arginyl-tRNA synthetase, glutaminyl-tRNA synthetase and glutamyl-tRNA synthetase in their mode of amino acid activation. The results suggest that an aaRS that does not normally require tRNA for amino acid activation can be switched to a tRNA-dependent mode.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRS) catalyze the esterification of an amino acid to the 3'-end of its cognate tRNA in a two-step reaction. In the first step, the amino acid is activated in the form of an enzyme-bound

aminoacyl-adenylate intermediate, while the second step involves the transfer of the amino acid to the 3'-end of its cognate tRNA to produce an aminoacyl-tRNA (1). Based on two distinct ATP-binding cores, the 20 aaRSs are equally divided into two classes (2,3). In most cases, formation of the aminoacyl-adenylate does not require the presence of its cognate tRNA and can be performed by the isolated catalytic domain of some synthetases (4,5). However, for three class I synthetases, arginyl-, glutaminyl- and glutamyl-tRNA synthetase (ArgRS, GlnRS and GluRS, respectively), as well as the exceptional class I lysyl-tRNA synthetase (LysRS), tRNAs are absolutely required to accomplish the first step of the reaction.

The requirement of tRNA in aminoacyl-adenylate formation has been confirmed for ArgRS from different sources (6–11) and is likely a common feature of this enzyme. An intact 3'-terminal adenosine of its cognate tRNA is also required, because arginine activation cannot be induced by periodate-treated tRNA (7,9,10,12,13). Proper tRNA structure is also necessary for arginine activation (13). For GluRS and GlnRS, amino acids also cannot be activated without their cognate tRNA (14–17). The activator function also requires the integrity of the 3' terminus of the tRNA, and chemical modification of this terminus abolishes this activity (14,16,18). Exceptionally, several bacteria and archaea have the class I-type LysRS instead of the class II-type LysRS (19,20). Class I LysRS is structurally related to GluRS (21), and does not catalyze lysyl-adenylate formation in the absence of tRNA (22). However, the biological significance of requiring tRNA to activate amino acids remains puzzling. The fact that ArgRS requires its cognate tRNA to activate arginine has led to the suggestion that the arginyl-adenylate intermediate does not exist in the arginylation reaction (23), although Kern and Lapointe (24,25) later demonstrated that GluRS charges tRNA with glutamate in two steps and that the glutamyl-adenylate intermediate exists, despite the fact that tRNA is needed for the first step.

*To whom correspondence should be addressed. Tel: 0086 21 54921222; Fax: 0086 21 5492 1011; Email: yxjin@sibs.ac.cn

Tryptophanyl-tRNA synthetase (TrpRS) is considered a class I aaRS, and together with all other synthetases except for ArgRS, GluRS, GlnRS and class I LysRS, can activate an amino acid substrate smoothly in the absence of cognate tRNA (1). We recently showed that the ATP-Pi exchange reaction of a mutated *Bacillus subtilis* TrpRS can be modulated by its inactivated tRNA^{Trp} (26).

Eukaryotic TrpRS differs from prokaryotic TrpRS in containing an appended N-terminus. For human TrpRS, the N-terminal domain is composed of an appended peptide and an eukaryote-specific patch (E82–K154) containing a β 1– β 2 hairpin structure adjacent to the ATP-binding pocket and the KMSAS loop (27). Deletion of this hairpin severely reduces aminoacylation activity, suggesting that human TrpRS has a more complicated aminoacylation mechanism than *B. subtilis* TrpRS. At the β 1– β 2 hairpin of human TrpRS (1R6T from the Protein Data Bank), the backbone of V85 and V90 form a hydrogen bond. The side chains of the two valines protrude into the catalytic domain, which looks like a buckhorn (Figure 1A).

In this study, we mutated V85 and V90 and unexpectedly found that the V85S mutant could no longer activate tryptophan even though its tryptophanylation activity still proceeded normally. Furthermore, we confirmed that the tryptophan activation of V85S can be partially rescued by

the addition of bovine tRNA^{Trp}. We constructed further mutants at V85 to further investigate and found that the V85E mutation also caused a switch to a tRNA-dependent mode of amino acid activation, just like ArgRS, GluRS, GlnRS and class I LysRS. Structural analysis showed that V85 can interact with I311 through a hydrophobic interaction to affect tryptophan activation, which was confirmed by I311 mutations. The results indicate that an aaRS that does not normally require tRNA for amino acid activation can be switched to a tRNA-dependent mode.

MATERIALS AND METHODS

Strains and plasmids

Bovine tRNA^{Trp} was expressed in *Escherichia coli* JM109 and human TrpRS and its mutants were expressed in *E. coli* BL21-CodonPlus (DE3)-RIL. BL21-CodonPlus (DE3)-RIL was purchased from Stratagene (La Jolla, CA, USA). The bovine tRNA^{Trp} gene was cloned into the pGEM-9Zf (–) plasmid obtained from Promega (Madison, WI, USA) and was a kind gift from Dr Xue Hong at the Hong Kong University of Science and Technology. The plasmid pTrec-hTrpRS containing the human *trpS* gene was also a gift from Dr Xue Hong.

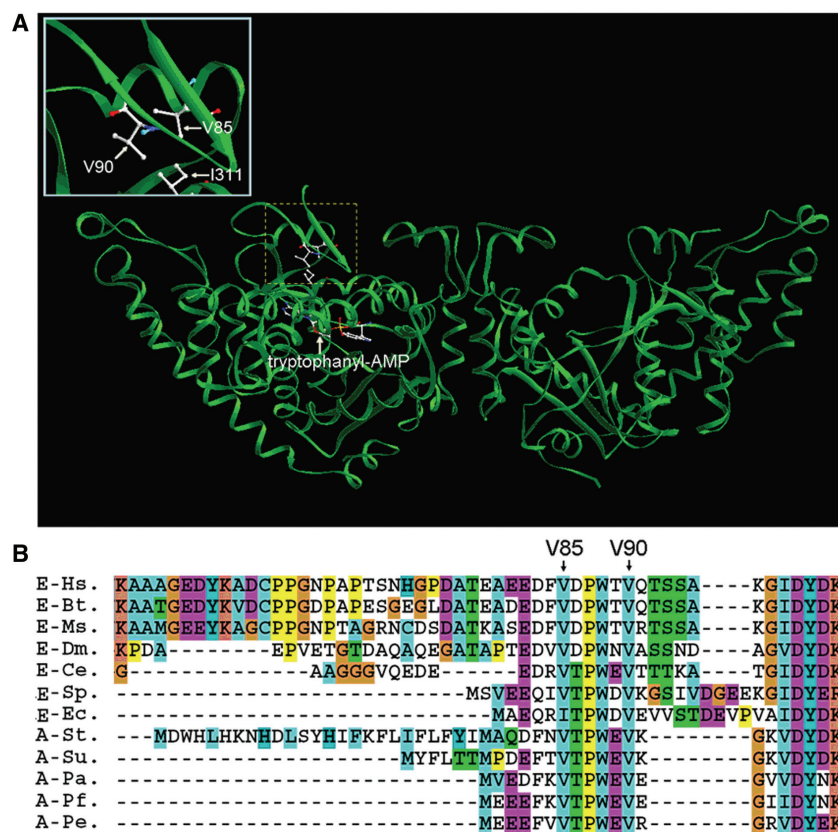


Figure 1. Primary and tertiary structure of human TrpRS. (A) Crystal structure of full-length human TrpRS (40). The tryptophanyl-AMP intermediate is indicated by an arrowhead. The V85 and I311 interface between the β 1– β 2 hairpin and main body is enlarged in the boxed inset. (B) Primary sequence alignment of the region of human TrpRS containing the β 1– β 2 hairpin. The conserved V85 and V90 in TrpRSs are indicated by arrowheads.

The human *trpS* gene expression vector pET24a (+) was purchased from Novagen.

Sequence homology analysis and structure comparison

TrpRS protein sequences from archaeobacteria and eukaryotes were downloaded from the aaRS database (www.pozman.edu.pl/aars) (28). Abbreviations of the species are as follows: E-Hs, *Homo sapiens*; E-Bt, *Bos taurus*; E-Ms, *Mus musculus*; E-Dm, *Drosophila melanogaster*; E-Ce, *Caenorhabditis elegans*; E-Sp, *Schizosaccharomyces pombe*; E-Ec, *Encephalitozoon cuniculi*; A-St, *Sulfolobus tokodaii*; A-Su, *Sulfolobus solfataricus*; A-Pa, *Pyrococcus abyssi*; A-Pf, *Pyrococcus furiosus*; A-Pe, *Pyrobaculum aerophilum*. The first letter E in the abbreviation means eukaryote, and A means archaea. Multiple sequence alignments were carried out with Clustal X 1.83 software (ftp-igbmc.u-strasbg.fr/pub/ClustalX/). The pdb files of human TrpRSs (1O5T, 1R6T, 1ULH and 2DR2) were downloaded from the Protein Data Bank (<http://www.rcsb.org/pdb/>). Structure comparisons and figure production were carried out with Swiss-PdbViewer 3.7 software, which was downloaded from <http://www.expasy.ch/spdbv/text/download.htm>. The operation pathway of Swiss-PdbViewer 3.7 software for structure comparison was Fit\Magic Fit\CA (carbon alpha) only).

Expression and purification of human TrpRS and bovine tRNA^{Trp}

Expression and purification of human TrpRS were carried out as described previously (29). Concentrations of purified proteins were determined with the Bradford reagent (30). Mutagenesis was carried out by PCR as described previously (31). Mutant human *TrpRS* genes were verified by DNA sequencing. Abbreviations of the mutations were as follows: V85A, valine 85 to alanine; V85E, valine 85 to glutamate; V85K, valine 85 to lysine; V85L, valine 85 to leucine; V85S, valine 85 to serine; V90A, valine 90 to alanine; V90S, valine 90 to serine; V85A/V90A, V85A/V90A double mutations; I311V, isoleucine 311 to valine; I311E, isoleucine 311 to glutamate. The bovine tRNA^{Trp} used for the enzyme activity assay was expressed and purified as described previously (32).

Periodate oxidation of tRNA

The oxidation of tRNA by sodium periodate was carried out as previously described (33,34) with some modifications. Bovine tRNA^{Trp} was treated with 10 mM sodium periodate in 1 ml of 150 mM sodium acetate (pH 5.3), incubated at 4°C in the dark for 1 h. Glucose was then added to a final concentration of 10 mM to remove excess sodium periodate. The tRNA was incubated at 37°C for 30 min and then precipitated with ethanol.

Enzymatic assays

The ATP-PPi exchange reaction was assayed at 30°C in a reaction mixture containing 100 mM Tris-HCl pH 7.8, 10 mM potassium fluoride, 10 mM magnesium chloride,

4 mM ATP, 0.02 μCi [γ -³²P]ATP, 4 mM sodium pyrophosphate and 2 mM tryptophan in total volume of 20 μl. Reactions were initiated by the addition of 0.2 μM enzyme. At each time point, samples were quenched on ice. One microliter of the reaction mixture was spotted onto PEI cellulose TLC plates (purchased from Merck, Germany) and developed in 1 M KH₂PO₄ and 1 M urea to separate the ATP and PPi. The radioactivity was revealed and quantified using a PhosphorImagerTM (Molecular Dynamics, Little Chalfont, Bucks, UK).

For mutants V85S, V85E, V85K, V85A/V90A and I311E, the tRNA-dependent ATP-PPi exchange reaction was assayed. Wild-type bovine tRNA^{Trp} and oxidized bovine tRNA^{Trp} were added to the PPi exchange reaction mixture to a final concentration of 60 μM. The other components for the reaction were identical to that described above.

The aminoacylation assay was also carried out at 30°C in a mixture containing 4 mM ATP, 0.8 mM DTT, 1 μCi L-[5-³H]tryptophan, 12 μM L-tryptophan, 8 mM MgCl₂, 80 mM Tris-HCl, pH 7.5 and 12 μM purified bovine tRNA^{Trp} in a total volume of 50 μl. Reactions were initiated by the addition of 50 nM enzyme. At each time point, samples were quenched on ice and 20 μl aliquots were spotted onto filter paper disks, which were washed three times with ice-cold 5% trichloroacetic acid containing 0.05% tryptophan and with cold anhydrous ethanol, dried and transferred into vials for determination of radioactivity. For all kinetic assays, the concentration of tRNA^{Trp} varied from 0.2 to 12.8 μM. Each reaction was repeated at least four times under the same conditions. The k_{cat}/K_m for aminoacylation was calculated from Eadie-Hofstee plots. All data were fitted to the Michaelis-Menten equation.

RESULTS

The V85S mutation enables human TrpRS to switch to tRNA-dependent tryptophan activation

Based on a structural analysis of human TrpRS, we found that there were two adjacent valines in the middle of the β1 and β2 sheet, V85 and V90, whose side chains both approach the substrate-binding pocket (Figure 1A). The β1-β2 hairpin is essential to the aminoacylation activity of human TrpRS (27), and therefore these two valine residues may be important for enzyme activity. Based on sequence alignment, we found that V85 and V90 are conserved in eukaryotes and archaeobacteria (Figure 1B). Therefore, we constructed mutations at these two positions, V85 and V90, to generate the V85A, V85S, V90A and V90S variants.

In the ATP-PPi exchange reaction, the V85A, V90A and V90S mutants showed decreased, but visible tryptophan activation activity, with V85A having the lowest enzyme activity (Figure 2A). Furthermore, when V85 was mutated to serine, no ATP-PPi exchange activity was detected, even at a very high enzyme concentration (5.1 μM) (Figure 2A). Unexpectedly, the aminoacylation activity of the V85S mutant remained comparable to the other three mutant enzymes (Figure 2B) and could not catalyze the tryptophanylation reaction in the absence of

ATP (Figure 2B). This suggested that the aminoacylation reaction of the *V85S* mutant proceeds normally and that the mutation does not change the recognition of ATP. These results implied that the *V85S* mutant might be able to activate tryptophan only in the presence of tRNA. To investigate whether the *V85S* mutant assumes a tRNA-dependent ATP-PPi exchange activity, wild-type or sodium periodate-treated bovine tRNA^{Trp} was added to the ATP-PPi exchange reaction mixture. Unexpectedly, both the wild type and the sodium periodate-treated bovine tRNA^{Trp} were able to partially rescue the ATP-PPi exchange activity of the *V85S* mutant (Figure 2C).

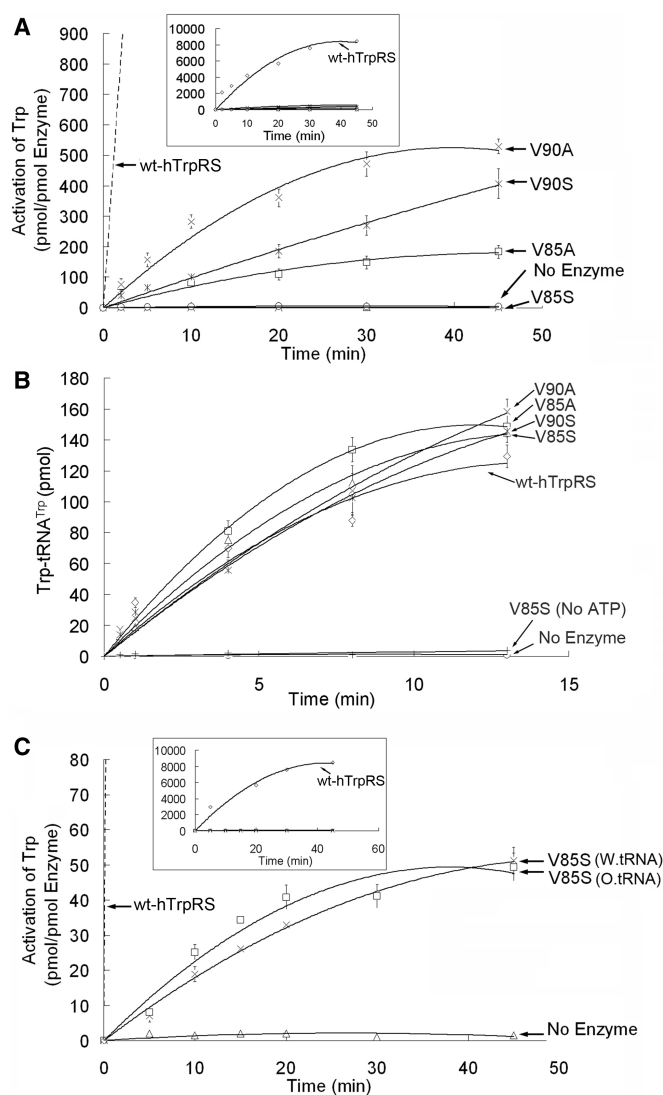


Figure 2. Enzymatic activities of the *V85A*, *V85S*, *V90A* and *V90S* mutants. (A) ATP-PPi exchange activity of the four mutants and wild-type human TrpRS. To display the whole curve of wt-hTrpRS, we enlarged the scale of the Y-axis in the boxed inset. (B) Aminoacylation activity of the four mutants and wild-type human TrpRS. The enzyme concentrations are 5 nM for wild-type human TrpRS (wt-hTrpRS), 20 nM for *V85A* and *V90A* and 50 nM for *V85S* and *V90S*. (C) PPi exchange activity of the *V85S* mutant in the presence of wild-type or oxidized bovine tRNA^{Trp}. The wt-hTrpRS curve is shown in the boxed inset.

Sodium pyrophosphate can greatly inhibit the tryptophanylation reaction. After adding the wild-type tRNAs to the ATP-PPi exchange reaction, the tryptophanyl-tRNA^{Trp} could hardly be detected (data not shown). Thus, the tRNA-independent ATP-PPi exchange reaction for human TrpRS is switched to a tRNA-dependent mode by the single *V85S* mutation. However, in contrast to ArgRS, GlnRS and GluRS (7,9,10,12–14,16,18), the integrity of the 3' terminus of the tRNA was not necessary for the ATP-PPi exchange activity of the *V85S* mutant (Figure 2C). Therefore, it is reasonable to conclude that human TrpRS is switched to a 'nonproductive' form by *V85S* mutation but can be induced to a 'productive' form by tRNA binding. Comparing Figure 2A B, we conclude that V85 mutations have a greater impact on tryptophan activation than the tryptophanylation reaction.

Additional mutants of valine 85

To further investigate the role of the V85 residue in the ATP-PPi exchange reaction, we constructed three more mutations at this position including *V85L*, *V85E* and *V85K*. Relative to the *V85E* and *V85K* mutations, the *V85L* mutant did not show a large decrease in tryptophan activation (Figure 3A). However, the *V85E* and *V85K* mutants lacked the ATP-PPi exchange activity of human TrpRS under normal conditions (Figure 3A), just like the *V85S* mutant (Figure 2A). In the tryptophanylation reaction, the *V85L* mutant was able to acylate bovine tRNA^{Trp} with very high efficiency (Figure 3B). Similar to the *V85S* mutant, the *V85E* mutant has low but noticeable activity (Figure 3B). In contrast, the *V85K* mutant had barely detectable aminoacylation activity (Figure 3B). In the presence of oxidized bovine tRNA^{Trp}, the *V85E* TrpRS was unable to catalyze the ATP-PPi exchange reaction, but the wild-type bovine tRNA^{Trp} could still serve as an activator (Figure 3C), which suggests that the hydroxyl groups of the 3' adenosine of tRNA can also activate human TrpRS. For the *V85K* mutant, wild-type tRNA could slightly rescue its PPi exchange activity while tRNA treated with sodium periodate could not (Figure 3C).

Thus, we conclude that mutation of V85 to a hydrophilic amino acid such as serine, lysine and glutamate abolishes the normal ATP-PPi exchange activity of human TrpRS (Figures 2A and 3A). On the other hand, replacing V85 with a neutral amino acid such as alanine, or a hydrophobic amino acid such as leucine, did not produce the same result (Figures 2A and 3A). Moreover, the more hydrophobic the side chains at the V85 position, the higher the enzyme activity.

Interaction between the $\beta 1$ – $\beta 2$ hairpin and tRNA

The *V85S* single mutation switches human TrpRS from a tRNA-independent to a tRNA-dependent mode of tryptophan activation (Figure 2). For the *V85S* mutant, the tRNA molecule, not the hydroxyl group at the 3' end, serves as an activator, which is different from ArgRS, GluRS and GlnRS (Figure 2C). The *V85E* mutant was activated to catalyze the ATP-PPi exchange reaction only in the presence of wild-type bovine tRNA^{Trp} (Figure 3C).

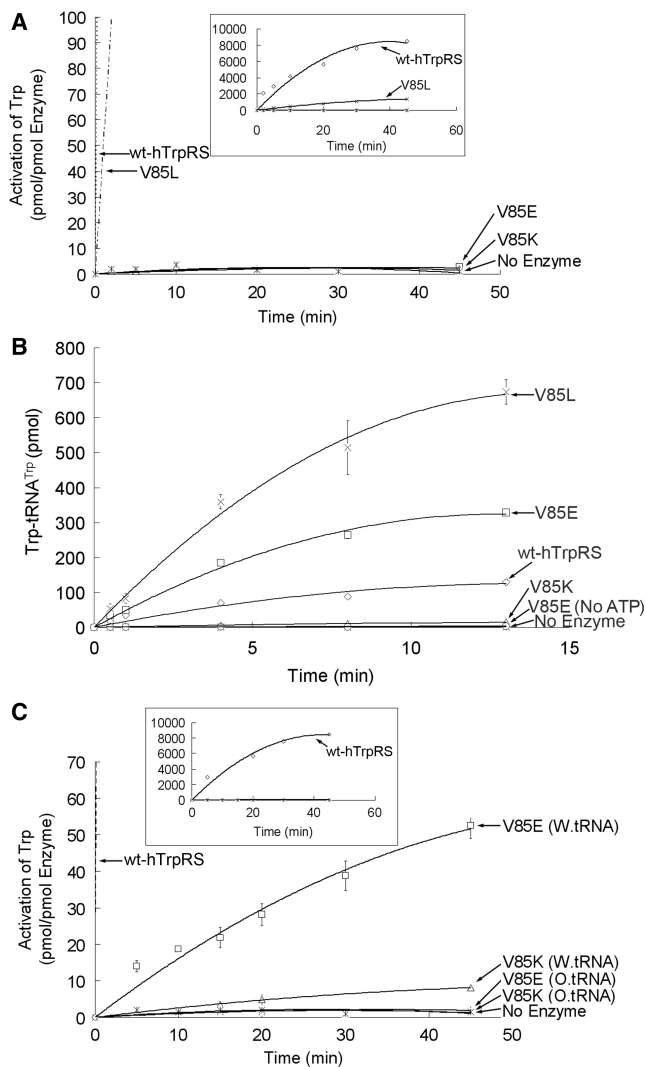


Figure 3. Enzymatic activities of the *V85E*, *V85K* and *V85L* mutants. (A) ATP-PPi exchange activity of the three mutations and wild-type human TrpRS. The boxed inset displayed the whole curve of wt-hTrpRS and the *V85L* mutant. (B) Aminoacylation activity of the three mutations and wild-type human TrpRS. The enzyme concentrations are 5 nM for wt-hTrpRS and *V85L* and 200 nM for *V85E* and *V85K*. (C) PPi exchange activity of the *V85E* and *V85K* mutants in the presence of wild-type or oxidized bovine tRNA^{Trp}. The enlarged scale for wt-hTrpRS and the *V85L* mutant are shown in the boxed inset.

This suggests that the 2' or 3' hydroxyl group at the 3' adenosine of tRNA also can act as an activator, similar to ArgRS, GluRS and GlnRS. The *V85K* mutant had barely detectable enzyme activity.

Based on the recent co-crystal structure of human TrpRS and bovine tRNA, we concluded that V85 should be close to a tRNA acceptor stem when tRNA is bound to human TrpRS (27,35). Thus, the phosphate groups of tRNAs should be easily affected by the V85 mutation. To verify our hypothesis, we calculated the K_m of tRNA for the *V85S*, *V85E* and *V85K* mutants. For the *V85S* mutant, the K_m value was 2.14 μM , similar to 1.30 μM for wild-type human TrpRS (Table 1). The K_m value was 8.50 μM for *V85E* and 0.294 μM for *V85K* (Table 1). Therefore, it is reasonable to conclude that the *V85S* mutant that leaves the side chain uncharged has little effect on tRNA binding, so not only wild type but also oxidized tRNA is able to properly bind to the enzyme to prompt the ATP-PPi exchange reaction. Because the side chain of glutamate is negatively charged, the *V85E* mutation would push out the negatively charged phosphate groups of tRNAs, weakening tRNA binding. The *V85K* mutant, with a positively charged side chain, can attract the negatively charged phosphate groups of tRNAs. As a result, tRNA may be bound to human TrpRS at an incorrect position when V85 was mutated to a lysine. Therefore, the fact that the *V85K* mutant has the lowest acceptor activity is reasonable.

We next calculated the K_m for tRNA and the k_{cat} values of all the above mutations (Table 1). These varied dramatically at the $\beta 1$ - $\beta 2$ hairpin, with K_m ranging from 0.294 to 24.8 μM and k_{cat} ranging from $9.44 \times 10^{-4} \text{ s}^{-1}$ to 5.11 s^{-1} . The K_m values suggest that V85 and V90 can interact with tRNA when tRNA binds to human TrpRS. Thus, it is logical that charge variations of the side chains at V85 and V90 have the greatest impact on tRNA acceptor activity.

V85 interacts with I311 of the AIDQ sequence via by hydrophobic side chains

Based on the crystal structure of the full-length human TrpRS (1R6T), we found that V85 is far away from the substrate-binding pocket (Figure 1A) and therefore could not directly affect tryptophan activation. However, the side chain of V85 is only 4.41 Å away from the side chain

Table 1. The aminoacylation kinetics constant for wt-hTrpRS, *V85A*, *V85S*, *V90A*, *V90S*, *V85E*, *V85K*, *V85L*, and *V85A/V90A* mutants. All K_m are for tRNA

Enzymes	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	Relative activity
wt-hTrpRS	1.30 ± 0.16	1.37 ± 0.14	1.05	1.00 ^a
<i>V85A</i>	1.41 ± 0.15	0.476 ± 0.026	0.338	0.322
<i>V85S</i>	2.14 ± 0.54	$(2.77 \pm 0.77) \times 10^{-2}$	1.29×10^{-2}	1.23×10^{-2}
<i>V90A</i>	18.4 ± 2.2	1.39 ± 0.070	7.55×10^{-2}	7.19×10^{-2}
<i>V90S</i>	5.20 ± 0.90	$(6.25 \pm 0.63) \times 10^{-2}$	1.20×10^{-2}	1.14×10^{-2}
<i>V85E</i>	8.50 ± 1.4	$(1.97 \pm 0.37) \times 10^{-2}$	2.32×10^{-3}	2.21×10^{-3}
<i>V85K</i>	0.294 ± 0.047	$(9.44 \pm 2.4) \times 10^{-4}$	3.21×10^{-3}	3.06×10^{-3}
<i>V85L</i>	24.8 ± 5.6	5.11 ± 0.99	0.206	0.196
<i>V85A/V90A</i>	2.23 ± 0.24	$(4.71 \pm 0.49) \times 10^{-2}$	2.11×10^{-2}	2.01×10^{-2}

^aThe activity of wild-type human TrpRS was set to 1.00.

of I311 (Figure 1A). I311 is a residue in the conserved AIDQ sequence. A310 and D312 interact with the ribose of ATP through hydrogen bonds to stabilize ATP binding (35). Combining all findings, we concluded that V85 may affect I311 through hydrophobic interactions between their side chains to affect tryptophan activation. Furthermore, we compared the crystal structure of unliganded human mini-TrpRS (1ULH), which contains the $\beta 1$ – $\beta 2$ hairpin structure at one monomer, with the unliganded crystal structure of T2-TrpRS (1O5T) in which the $\beta 1$ – $\beta 2$ hairpin structure is hydrolyzed. Both the mini-TrpRS and T2-TrpRS are truncated forms of human TrpRS at the N-terminus with mini-TrpRS encoding residues 48–471 and T2-TrpRS encoding residues 94–471. We found that the AIDQ sequence of mini-TrpRS assumes a compact form (Figure 4A), while the AIDQ sequence of T2-TrpRS adopts a loose form (Figure 4A). When bovine tRNA^{Trp} binds to T2-TrpRS (2DR2), as expected, its AIDQ sequence switches to the compact form (Figure 4B). Therefore, it is reasonable to conclude that, at least partially, the interaction of V85 with I311 can induce the AIDQ sequence to an active compact form. If V85 is mutated to a hydrophilic amino acid that disrupts the hydrophobic interaction between V85 and I311 (such as serine, glutamate and lysine), the conformational change in the AIDQ sequence might not be induced correctly. Alternatively, because the 3' end of tRNA and the AIDQ sequence interact (35), tRNA can substitute for the function of the $\beta 1$ – $\beta 2$ hairpin.

To confirm our hypothesis, we mutated I311 to valine and glutamate and determined their enzyme activity. As we had expected, when the isoleucine was mutated to acidic glutamate, the normal ATP-PPi exchange activity of human TrpRS was abolished (Figure 5A), but the mutant enzyme could still catalyze the tryptophanylation reaction with decreased efficiency (Figure 5B). Wild-type bovine tRNA^{Trp}, not the oxidized form, was able to promote the ATP-PPi exchange reaction (Figure 5C), just like the V85E mutation. Compared with wild-type human TrpRS, the I311V mutation had little effect on both the ATP-PPi exchange reaction and tryptophanylation reaction relative to the large effect of the *I311E* mutant on both reactions (Figure 5A and B). Therefore, the above inference should be true. Relative to the *V85E* mutant, the *I311E* mutant has even lower aminoacylation activity. The *I311E* mutant might interfere with the interaction between the A76 ribose of bovine tRNA^{Trp} and AIDQ sequence (35).

The V90 mutation enhances the hydrophobic interaction between V85 and I311

We have found that the V90 mutations had little effect on the ATP-PPi exchange reaction. Mutants of human TrpRS at position 90 only showed reduced ATP-PPi exchange activity. Based on structural analysis of the full-length human TrpRS (1R6T), we found that the side chain of V90 is farther away from the side chain of I311 than that of V85. Thus, V90 should only act as a subsidiary residue to enhance the hydrophobic interaction between V85 and I311. Therefore, we constructed a double mutant

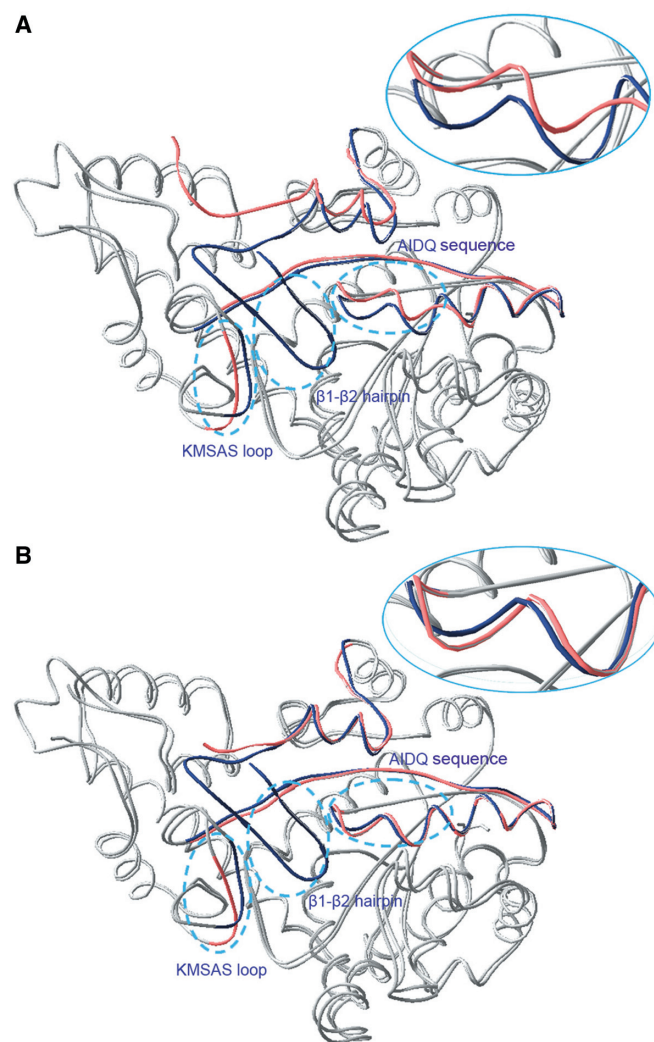


Figure 4. Structural comparisons of human T2-TrpRS and mini-TrpRS. (A) Comparison of unliganded T2-TrpRS (1O5T) and mini-TrpRS (1ULH). (B) Comparison of T2-TrpRS bound to bovine tRNA^{Trp} (2DR2) and mini-TrpRS (1ULH). For clarity, the tRNA molecule of 2DR2 is not displayed, and only part of the N-terminus, the AIDQ sequence, and the KMSAS loop are colored dark blue for mini-TrpRS and pink for T2-TrpRS. In both Figure 4A and B, the $\beta 1$ – $\beta 2$ hairpin, the AIDQ sequence and the KMSAS loop are indicated with sky blue dashed ellipses. The conformational change of the AIDQ sequence is enlarged in the circled ellipse inset.

of human TrpRS, *V85A/V90A*. As expected, the behavior of *V85A/V90A* was similar to the *V85S* mutant (Figure 6). We also calculated the K_m and k_{cat} for the *V85A/V90A* double mutant, which were 2.23 μ M and 0.0471 S^{-1} , respectively (Table 1).

There are no Schiff bases formed between oxidized bovine tRNA^{Trp} and human TrpRS mutants

Previous studies have shown that tRNA oxidized by sodium periodate can form Schiff bases with the ϵ -NH₂ of lysine residues close to the binding site (33,36), irreversibly inhibiting enzyme activity. To clarify whether the oxidized bovine tRNA^{Trp} can form Schiff bases with human TrpRS, we pre-incubated the *V85E*, *V85K*, *V85S*,

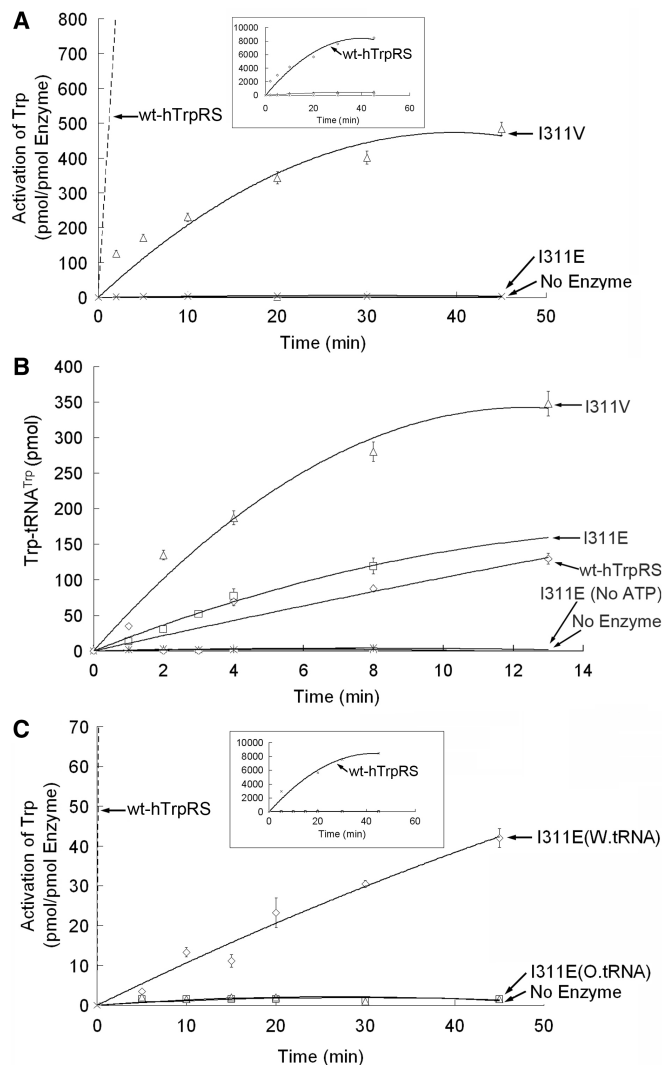


Figure 5. Enzymatic activities of the *I311E* and *I311V* mutants. (A) ATP-PPi exchange activity of the two mutants and wild-type human TrpRS. The boxed inset displayed the whole curve of wt-hTrpRS. (B) Aminoacylation activity of the two mutants and wild-type human TrpRS. The enzyme concentrations are 5 nM for wild-type human TrpRS, 50 nM for the *I311V* mutant and 200 nM for the *I311E* mutant. (C) PPI exchange activity of the *I311E* mutant in the presence of wild-type or oxidized bovine tRNA^{Trp} and wild-type human TrpRS. The enlarged scale for wt-hTrpRS is shown in the boxed inset.

V85A/V90A and *I311E* mutants with oxidized tRNA for 10 min, 20 min, 30 min and 40 min, and then performed the aminoacylation assay (see Materials and Methods section for aminoacylation conditions). After 10 min, reactions were stopped and enzyme activity was determined. The results showed that oxidized tRNA did not inhibit the activity of the enzymes (Supplementary Figure S1). Therefore, Schiff bases do not form between oxidized tRNA and TrpRS.

DISCUSSION

ArgRS, GluRS, GlnRS and Class I LysRS all share the common feature that is the absolute requirement of tRNA

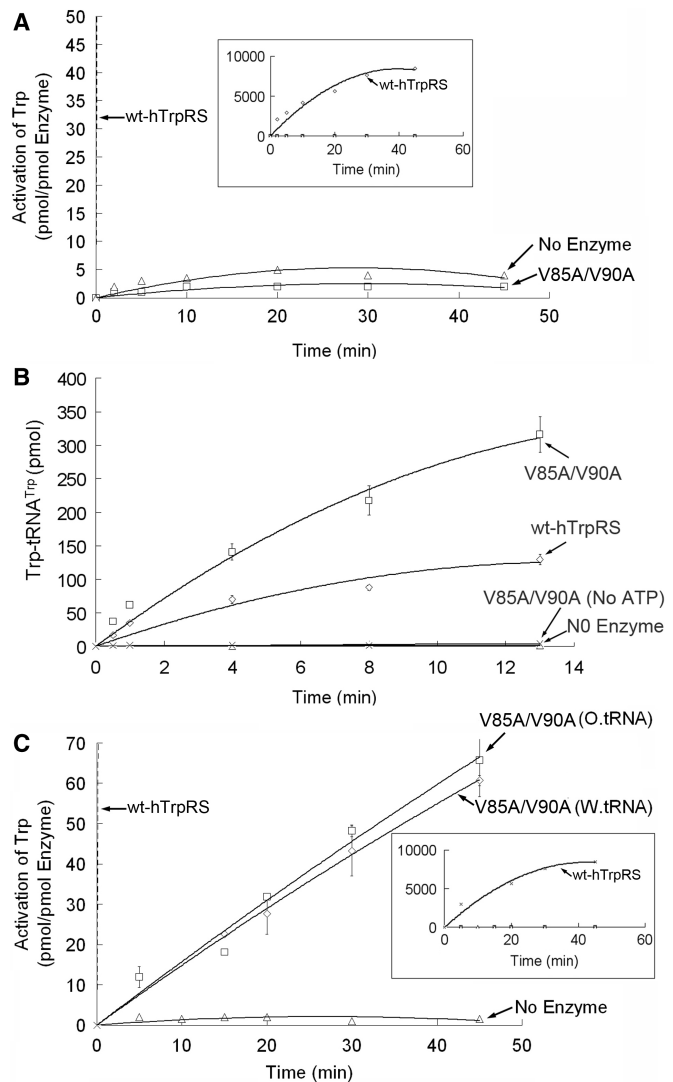


Figure 6. Enzymatic activities of the *V85A/V90A* double mutants. (A) ATP-PPi exchange activity of this mutant and the wild-type human TrpRS. The enlarged scale is shown in the boxed inset for wt-hTrpRS. (B) Aminoacylation activity of the *V85A/V90A* double mutant and wild-type human TrpRS. The enzyme concentrations are 5 nM for wild-type human TrpRS and 200 nM for the *V85A/V90A* mutant. (C) PPI exchange activity of the *V85A/V90A* double mutant in the presence of wild-type or oxidized bovine tRNA^{Trp} and wild-type human TrpRS. The wt-hTrpRS curve is shown in the boxed inset.

in the amino acid activation step. For these aaRSs, the tRNA is postulated to serve as the enzyme activator in the first step and as the substrate in the second step of aminoacylation. Except for these four enzymes, the other class I aaRSs do not require the presence of tRNA to activate amino acid, such as TrpRS. Here, we combined mutations to illustrate that human TrpRS can be switched to a tRNA-dependent mode to activate tryptophan by single or double mutation(s) in the $\beta 1$ - $\beta 2$ hairpin and AIDQ sequence. These mutant enzymes were all tested at a high concentration ($>2.5 \mu\text{M}$) and were unable to activate tryptophan in the absence of tRNA. This finding was unexpected. These mutations abolished the PPI exchange activity of TrpRS, which can be partially rescued

by the addition of tRNA. For ArgRS, GluRS and GlnRS, comparisons of the tRNA-free and tRNA-bound enzyme crystals have revealed tRNA-induced enzyme conformational changes (37–39), which should be responsible for amino acid activation. The situation for human TrpRS should be the same and was confirmed by structural comparison (Figure 4). On the other hand, for wild-type human TrpRS, V85 should perform the same function, at least partially, as tRNA in the tryptophan activation reaction (Figure 4).

Conformational changes in human TrpRS induced by V85 would presumably require specific molecular interactions. We have identified an interface between the eukaryotic-specific patch and the catalytic center, which might be responsible, at least in part, for molecular interactions that would facilitate tryptophan activation. This site is defined in human TrpRS by the eukaryotic-specific patch that contains V85 at the $\beta 1$ – $\beta 2$ hairpin and I311 at the conserved AIDQ sequence which directly interacts with ATP, based on the crystal structure of full-length human TrpRS (40). The side chains of V85 and I311 are as little as 4.41 Å apart. A comparison of the X-ray crystal structures of T2-TrpRS and mini-TrpRS (both not bound by substrates) illustrates that the $\beta 1$ – $\beta 2$ hairpin is responsible for the ‘productive’ form of human TrpRS (Figure 4). We concluded that, at least partially, the compact form of the AIDQ sequence of human TrpRS is ensured by hydrophobic interactions between the side chains of V85 and I311, because the side chain of V90 is much farther away from the side chain of I311 than that of V85. V90 should be a minor residue to interact with I311 by hydrophobic interaction, which was confirmed by the *V85A/V90A* double mutant. A310 and D312 are known to be able to form hydrogen bonds with the ribose of ATP to stabilize ATP binding. Therefore, if V85 is mutated to a hydrophilic amino acid, the hydrophobic interaction between V85 and I311 will be disrupted and the AIDQ sequence might assume a loose form. As a result, the hydrogen bond interaction between ATP and the AIDQ sequence will be disrupted and ATP may not be able to bind TrpRS stably. Thus, mutated human TrpRS cannot catalyze tryptophan activation until tRNA is also bound.

For V85E and I311E, the oxidized tRNA cannot rescue their PPi exchange activity, but wild-type tRNA still can (Figures 3C and 5C). After treatment with sodium periodate, the 2′ and 3′ hydroxyl groups of tRNA are oxidized to aldehyde groups. This should not result in any large changes in the hydrogen bonds between the A76 of tRNA and residues D312 and Q313 of the AIDQ sequence (35), otherwise the tRNA oxidized by sodium periodate would not be able to bind to human TrpRS properly and rescue the PPi exchange activity of the *V85S* mutant and the *V85A/V90A* double mutants. These results imply that the 2′ or 3′ hydroxyl group also can serve as an activator, just like ArgRS, GluRS and GlnRS. As enzyme activity proceeds, the 2′ or 3′ hydroxyl group of A76 will consequentially interact with the Trp–adenylate intermediate to accept activated tryptophan. The interaction between tRNA and Trp–adenylate may stabilize ATP binding allowing the human TrpRS to activate

tryptophan, even though V85 or I311 was mutated to glutamate. Furthermore, the sequence alignments show that V85 and V90 are conserved in eukaryotes and archaeobacteria, including the proline and tryptophan in the $\beta 1$ – $\beta 2$ hairpin (Figure 1B). Therefore, the $\beta 1$ – $\beta 2$ hairpin structure of human TrpRS should be conserved in eukaryotes and archaeobacteria and its function should be the same as that in human TrpRS.

Wild-type human TrpRS or mini-TrpRS does not require its cognate tRNA to activate tryptophan (41). However, we can switch its tRNA-independent PPi exchange activity to a tRNA-dependent mode by introducing mutations within the eukaryote-specific patch (E82–K154), which is not found in prokaryotic enzymes. It raises the question of whether the tRNA-independent PPi exchange activity of human TrpRS is an ancestral feature or has been more recently acquired with the acquisition of the eukaryote-specific patch. Our results cannot give a definite answer, although it seems that the partition between tRNA-independent and tRNA-dependent PPi exchange activity within a single aaRS can shift dramatically with relative evolutionary ease. Based on sequence alignment, we recently found a TrpRS from *Pyrococcus horikoshii*, a hyper-thermophilic archaeobacterium. This TrpRS has eukaryotic features, but the eukaryotic-specific patch is completely absent in this enzyme. This archaeobacterial TrpRS could provide further evidence to define the evolutionary connection between TrpRS and ArgRS-like aaRSs.

For ArgRS, GluRS, GlnRS and class I LysRS, the biological significance of requiring tRNA as a cofactor to activate amino acid is unclear. However, a previous study showed that tRNA binding can help these four class I aaRSs specially recognize their cognate amino acid. The co-crystal structure of *Thermus thermophilus* GluRS and tRNA^{Glu} with or without glutamate demonstrated that the amino acid-binding site of *T. thermophilus* GluRS is incomplete and the presence of the cognate tRNA^{Glu} facilitates glutamate binding by the enzyme (39). In the absence of tRNA, *T. thermophilus* GluRS binds not only L-glutamate but also noncognate amino acids such as D-glutamate, L-aspartate and L-glutamine (42). The binding of the noncognate amino acids is eliminated by tRNA^{Glu} binding to the enzyme. Similarly, the co-crystal structure of *E. coli* GlnRS indicated that the tRNA itself is involved in the Gln-AMP-binding site (38). For ArgRS, the *Bacillus stearothermophilus* and *Neurospora crassa* ArgRSs can bind the cognate arginine only in the presence of tRNA^{Arg} (7,10). A similar phenomenon was also reported for class I LysRS (22). The recently discovered dual specificity enzyme, prolyl-tRNA synthetase (ProRS), can acylate not only proline but also cysteine, although it can only recognize cysteine in the presence of Trna (43,44). The existence of aaRSs that can catalyze the synthesis of more than one aminoacyl-tRNA is assumed to be an important step in the evolution of these enzymes (45). tRNA-dependent amino acid recognition may be a relic of synthetase evolution. However, a recent paper by Carter *et al.* (46) showed that the minimal TrpRS catalytic domain from *B. stearothermophilus* can still catalyze the tryptophan activation step without tRNA and also

mischarge tRNA. Therefore, the biological significance of tRNA-dependent amino acid recognition remains puzzling. Otherwise, is the tRNA-dependent amino acid recognition inherent to class I aaRSs? Maybe we can get the answers in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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