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Activities of 20 aminoacyl-tRNA synthetases expressed in a reconstituted translation system in *Escherichia coli*

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ABSTRACT

A significant challenge in the field of in vitro synthetic biology is the construction of a self-reproducing cell-free translation system, which reproduces its components, such as translation proteins, through translation and transcription by itself. As a first step for such construction, in this study we expressed and evaluated the activity of 20 aminoacyl-tRNA synthetases (aaRSs), a major component of a translation system, in a reconstituted translation system (PURE system). We found that 19 aaRS with the exception of phenylalanyl-tRNA synthetase (PheRS) are expressed as soluble proteins and their activities are comparable to those expressed in *Escherichia coli* . This study provides basic information on the properties of aaRSs expressed in the PURE system, which will be helpful for the future reconstitution of a self-reproducing translation system.

1. Introduction

PURE (Protein synthesis Using Recombinant Elements) system is a reconstituted cell-free translation system composed of purified translation factors from *Escherichia coli* [1]. The controllability of its composition is useful in several applications, such as the directed evolution of proteins, studies of translation systems, and the constitution of artificial cell-like systems (recently reviewed in Matsubayashi et al. [2]). A significant challenge using PURE system is the construction of a self-reproducing translation system in which all translation proteins and RNAs, which are presently supplied externally, are expressed from DNA in PURE system. Using this system, we can reproduce the translation system by supplying the DNA encoding all of the translation factors. Such a self-reproducing translation system would enhance the usefulness of the translation system by reducing the cost, and also contribute to the construction of an artificial life-like system [3,4,5].

To date, several proteins have been expressed in PURE system. Niwa et al. comprehensively expressed *E. coli* genes and investigated their solubility. They found that most of the proteins were solubilized by the addition of chaperons [6]. In contrast, the activities of proteins expressed in PURE system are limited to several proteins, such as DHFR [7], RNA replicase [8], GFP [9], antibody [10], EmrE [11], sec translocon [12], connexicin [13], lipid synthases [14], FtsE [15], α-hemolysin [16], luciferase [17], and DNA polymerization protein [18]. In addition, the activities expressed in PURE system are not compared to those expressed in the cell in most cases.

In this study, we expressed 20 aminoacyl-tRNA synthetases (aaRSs), one of the major protein families of translation factors, in PURE system, and we evaluated their solubility, and aminoacylation activity of the expressed aaRSs. We found that, with the exception of phenylalanyl-tRNA synthetase (PheRS), all aaRS are expressed in PURE system as soluble and active proteins, and their activity is comparable with that of proteins expressed in *E. coli*.

2. Materials and methods

2.1. Materials

The components of the standard customized PURE system used in this study, such as ribosomes, translation proteins, aaRSs, and T7 RNA polymerase, were expressed in *E. coli* and purified as previously described [19]. The composition of the PURE system was described in our previous study [20] except for omitting *E. coli* thioredoxin 2. DnaK mix (including DnaK, DnaJ, and GrpE of *E. coli*)

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and GroE mix (including GroEL and GroES of *E. coli*) were purchased from Gene Frontier (Japan).

2.2. Plasmid construction

The plasmids for the expression of LeuRS, MetRS, SerRS, and the β-subunit of PheRS were constructed by inserting each gene into pET21. The plasmids were named pET-LeuRS, pET-MetRS, pET-SerRS, and pET-PheRS-β, respectively. For the expression of other aaRS, plasmids constructed in a previous study were used [19]. For PheRS, the plasmid previously reported [19], which encoded both the α- and β-subunit, was mixed with pET-PheRS-β at a 7:3 ratio to make α and β-subunits with approximately the same molar ratio. The DNA fragments for the expression of each aaRS in the PURE system were prepared by PCR using the plasmids described above as templates and primer 3 and 4 for AlaRS, AsnRS, and ThrRS. Primers 1 and 2 were used for other aaRSs. The primer sequences are shown in Table S2.

2.3. Solubility assay

The DNA fragment (2.5 nM) encoding each aaRS was added to the standard PURE system, including T7 RNA polymerase (500 nM) and [^{35}S]-methionine (2.5 μCi/ul), and incubated at 37 °C for 1 h. The mixtures were centrifuged at 22 $k \times g$ for 30 min to separate the supernatants (S) and precipitates (P), which were dissolved in the loading buffer (50 mM Tris–HCl (pH 7.4), 2% SDS, 60 μM 2-mercaptethanol, 10% glycerol). The supernatants and the dissolved precipitates were subjected to SDS-PAGE following fixation in a solution of 10% methanol and 10% acetic acid for 10 min. Protein bands on the fixed gel were visualized by autoradiography (Typhoon FLA 7000, GE Healthcare).

2.4. Aminoacylation activity assay

The DNA fragment (2.5 nM) encoding one of the 20 aaRSs was mixed with the PURE system including T7 RNA polymerase (500 nM) and the indicated reduced concentration of the corresponding aaRS (Table S1). The other aaRS concentrations are the same as those in the standard PURE system. The mixture was incubated at 37 °C for 1 h for translation. An aliquot of the mixture was incubated in the aminoacylation assay solution (the corresponding isotope-labeled amino acid, *E. coli* tRNA (Roche), 4 mM ATP, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 40 mM KCl) for 3 min at 37 °C. As radioisotope labels, we used [^{35}S] for cysteine and methionine and [^{14}C] for the other amino acids. The concentrations of amino acids and tRNA in this solution are different for each aaRS as shown in Table S3. The aminoacylation assay solution (20 μl) was spotted on a paper filter (Whatman 3 mm) and washed with 5% trichloroacetic acid. The radioactivity remaining on the filter (i.e., in the acid-insoluble fraction) was measured with a liquid scintillation counter. To prepare the standard curves with known concentrations of the purified aaRSs shown in Fig. S1, we diluted each purified aaRS with the PURE system incubated without the DNA, and subjected to the aminoacylation assay.

To measure the aaRS concentrations translated in the PURE system, we also performed aaRS expression as described above except for using the PURE system containing [^{35}S]-Met (2.5 μCi/μl). Aliquots were subjected to SDS-PAGE followed by fixation and autoradiography as described above. The intensities of the bands depending on the DNA fragments were compared to those of the spots of known amounts of [^{35}S]-Met to quantify the amounts of the translated aaRS.

3. Results

3.1. Solubility of the expressed aaRSs

We attempted to evaluate the solubility of the 20 aaRSs of *E. coli* when expressed in PURE system. As a PURE system, we used a customized PURE system based on our previous study [21]. We first performed solubility assays of the aaRS expressed in the PURE system as schematically described in Fig. 1A. We incubated each DNA fragment (2.5 nM) encoding each aaRS in the PURE system containing [^{35}S]-Met for 1 h at 37 °C to express each aaRS. We centrifuged the mixtures at 22 $k \times g$ for 30 min to separate the insoluble precipitate (P) from the soluble fraction (S), and applied them to SDS-PAGE followed by autoradiography (Fig. 2). Most of the expressed aaRSs were detected in the soluble fraction at least partially with a relatively lower amount of the α-subunit of PheRS. The ratios of the soluble fraction to the total protein are shown in Table 1 (Solubility). It is notable that the PURE system used in this study contains Trigger factor (TF), one of the chaperons of *E. coli*, and the solubility of GlnRS, ProRS, and ThrRS is consistent with the previous report [6].

3.2. Aminoacylation activity of the expressed aaRSs

Next, we measured the aminoacylation activity of the aaRSs expressed in the PURE systems as schematically described in Fig. 1B. To measure the aminoacylation activity of the 20 aaRSs expressed in the PURE system, we first had to reduce the aaRS concentration included in the PURE system because the standard high aaRS concentrations in the PURE system affect the aminoacylation activity assay of the newly expressed aaRS as background noise. We prepared 20 PURE system solutions with a reduced amount of each of the 20 aaRS (the reduced aaRS concentrations are shown in Table S1), and mixed them with the DNA fragments encoding the corresponding aaRS genes. The solutions were incubated for 1 h at 37 °C to express each aaRS. We transferred aliquots (1/10–1/80) of the solution to the aminoacylation assay

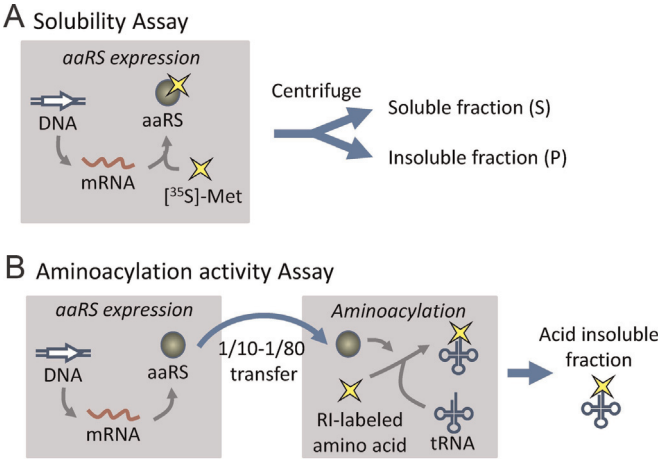

Fig. 1. Schematic drawings of experimental procedures. In this study, we performed two types of experiments examining solubility and aminoacylation activity of the aaRSs expressed in the PURE system. (A) Method for the solubility assay. We first expressed (by transcription and translation) each aaRS from DNA in the PURE system including [^{35}S]-Met for protein labeling, and then the reaction mixture was centrifuged to separate the soluble fraction (supernatant, S) from the insoluble fraction (precipitate, P). (B) Method for the aminoacylation activity assay. We first expressed each aaRS from DNA in the PURE system, and then an aliquot was transferred to the aminoacylation assay mixture including tRNA and each radioisotope-labeled amino acid ([^{14}C] or [^{35}S]). The radioactivity of the acid-insoluble fraction of this mixture, which included aminoacylated tRNA, was measured. In this assay, we also expressed each aaRS in the presence of [^{35}S]-Met to quantify the amount of translated aaRS.

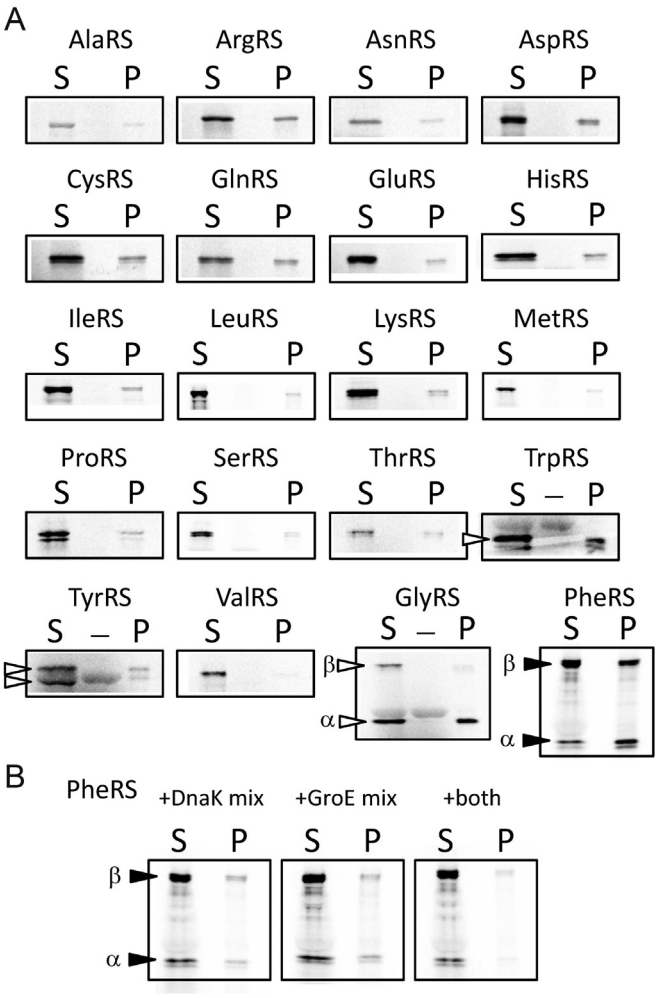

Fig. 2. Translation and solubility of 20 aaRS expressed in the PURE system. (A) After expression of each aaRS with [^{35}S]-methionine from the DNA fragments encoding each aaRS, the reaction mixtures were centrifuged at 22 $k \times g$ for 30 min, and supernatants (S) or precipitates (P) were subjected to SDS-PAGE followed by autoradiography. Parts of the gel images that contain major bands depending on the DNA fragments are shown. In the gel images for TrpRS, TyrRS, and GlyRS, bands depending on the DNA fragments are indicated (open arrows) as there are other bands that do not depend on the DNA fragments. For these three aaRSs, the results of the control experiments without the DNA fragments are also shown (–). (B) The effect of chaperon mixtures on the solubility of PheRS.

solution that contained the correspoinding radioisotope-labeled amino acid and tRNA. We incubated the solution for 3 min at 37 °C for aminoacylation of tRNA. The acid-insoluble aminoacylated-tRNA was captured on a filter pater and the radioactivity was measured with a liquid scintillation counter. By comparing this result with that obtained using a known concentration of aaRS protein, we evaluated the aminoacylation activity of the expressed aaRSs as a unit of molar equivalent of the purified aaRS protein (see Table 1, Aminoacylation activity). The aminoacylation activities of all aaRSs with the exception of PheRS were in the range of 54–3000 nM, which is approximately 600 times higher than the aaRS concentrations included in the initial PURE system on average. Note that in these experiments, we confirmed that the radioactivity in the acid-insoluble fraction was linearly correlated with the concentration of aaRS protein used for the aminoacylation assay (Fig. S1).

To evaluate the aminoacylation activity per protein, we next estimated the amount of translated aaRS in the PURE system. To estimate the translated aaRS concentration, we performed the same aaRS expression experiments as described in the previous paragraph except that [^{35}S]-Met was included, and after incubation the reaction mixtures were subjected to SDS-PAGE followed by autoradiography (Table 1, Translation). We divided the values of aaRS activity by the translated aaRS concentration to estimate the ratio of the activity of the aaRSs expressed in the PURE system to that expressed in and purified from *E. coli* (Table 1, Ratio of activity to translation). This ratio varied from 0.5 to 5.1 among the aaRSs, indicating that the activity of the 19 aaRS expressed in the PURE system was of the same order as that of the aaRS expressed in and purified from *E. coli*.

One of the possible reasons that PheRS activity was undetected when expressed in the PURE system is the lower solubility of its α-subunit (37% in Table 1). To examine this possibility, we added chaperon mixtures, reported to solubilize the α-subunit of PheRS [6], and measured the PheRS activity by the same method as described above. Consistent with a previous report [6], the band intensity of the α-subunit in the soluble fraction was increased by the addition of the chaperons (Fig. 2B), while the aminoacylation activity in the reaction mixture was still below the detection limit, indicating that the low solubility of PheRS was not the reason for the undetectable activity.

Table 1
Translation, aminoacylation activities, and solubility of 20 aaRS expressed in the PURE systems.

	Solubility (%)[a]	Aminoacylation activity (nM equivalent)[b]	Translation (nM)[b]	Ratio of activity to translation
AlaRS	88	310	230	1.3
ArgRS	89	460	290	1.6
AsnRS	92	150	50	3.0
AspRS	91	250	480	0.5
CysRS	93	1800	1500	1.2
GlnRS	86	250	350	0.7
GluRS	93	1500	350	4.3
GlyRS α	70	690	8900	1.3[c]
GlyRS β	88		540	
HisRS	90	3000	930	3.2
IleRS	93	310	87	3.6
LeuRS	98	770	150	5.1
LysRS	94	2700	860	3.1
MetRS	97	71	140	0.5
PheRS α	37	Not detected	210	
PheRS β	68		350	
ProRS	94	350	150	2.3
SerRS	95	240	71	3.4
ThrRS	84	54	23	2.3
TrpRS	82	1900	420	4.5
TyrRS	87	610	170	3.6
ValRS	95	96	23	4.1

[a] Quantified from the data shown in Fig. 2A.
[b] Measured with the reduced aaRS concentrations shown in Table S1. The activities are shown as a unit of molar equivalent of purified aaRS protein.
[c] Calculated based on the translation amount of the β-subunit of GlyRS (GlyRS β).

4. Discussion

In this study, we expressed 20 aaRSs of *E. coli* in the PURE system and evaluated their solubility and aminoacylation activity. We found that all aaRSs were expressed at least partly as soluble proteins, and we detected aminoacylation activity for all aaRSs with the exception of PheRS. The aminoacylation activity of the expressed aaRSs were of the same order as that of the aaRS expressed in and purified from *E. coli*. The amounts of the expressed aaRSs were approximately 600 times higher than the initial amount of aaRSs included in the PURE system on average, suggesting that the PURE system can synthesize sufficient amounts of

aaRSs, which is a requirement to achieve a self-reproducing translation system.

A remaining problem is the undetectable activity of PheRS expressed in the PURE system, which was possibly caused by inefficient formation of the active dimer. PheRS is a heterodimer containing an α- and a β-subunit, and the dimerization was reported to require a long incubation at a low temperature (4 °C) and a low salt strength (20 mM Tris–HCl) [22]. The reactions in this study were performed at 37 °C, and the PURE system used in this study contained a high salt concentration (280 mM glutamate potassium). The next important challenge is to find a condition under which all 20 aaRSs are expressed in their active forms; this might require the adjustment of salt and the compositions of other chemicals in the PURE system.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.bbrep.2015.08.006.

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