Reconstitution of 30S ribosomal subunits in vitro using ribosome biogenesis factors

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ABSTRACT
Reconstitution of ribosomes in vitro from individual ribosomal proteins provides a powerful tool for understanding the ribosome assembly process including the sequential incorporation of ribosomal proteins. However, conventional assembly methods require high-salt conditions for efficient ribosome assembly. In this study, we reconstituted 30S ribosomal subunits from individually purified ribosomal proteins in the presence of ribosome biogenesis factors. In this system, two GTPases (Era and YjeQ) facilitated assembly of a 30S subunit exhibiting poly(U)-directed polyphenylalanine synthesis and native protein synthesis under physiological conditions. This in vitro system permits a study of the assembly process and function of ribosome biogenesis factors, and it will facilitate the generation of ribosomes from DNA without using cells.

Keywords: biogenesis factors; cell-free translation system; in vitro reconstitution; ribosome; synthetic biology

INTRODUCTION
Ribosomes are large macromolecules that play a central role in protein synthesis in cells. Their three-dimensional structures were unveiled by a series of crystallographic studies (Ramakrishnan 2002) illustrating the complex of 16S rRNA and Group I (S4, S7, S8, S15, S17, and S20), Group II (S5, S6, S9, S11, S12, S13, S16, S18, and S19), and Group III proteins (S2, S3, S10, S14, and S21). In the reconstitution experiments, the existence of a reconstitution intermediate (RI) with distinctive properties has been demonstrated (Held et al. 1973). The RI, composed of 16S rRNA and 15 ribosomal proteins, cannot proceed to the maturation step without heat treatment at temperatures exceeding 40°C. The change of sedimentation of the particles suggested that heat activation induces a conformational change in the RI, resulting in active 30S subunit formation. The hierarchical structure of the assembly map of 30S ribosomal subunits is a coarse-grained view under equilibrium conditions, and recent kinetic studies illustrated the presence of several parallel assembly pathways (Mulder et al. 2010; Woodson 2011). Together with these results, the existence of the RI as confirmed by earlier studies suggests the presence of a bottleneck state or kinetic trap necessitating activation energy for conformational changes. These aspects make in vitro reconstitution difficult and inefficient.

In contrast to in vitro reconstitution, ribosome assembly proceeds via an extremely rapid and efficient process that is believed to require <3 min in the exponential growth phase (Champney 1977). In cells, biogenesis factors and modification enzymes change the local conformation of the precursor 30S subunits. The local conformation change may facilitate the transition to a more energetically stable state (Talkington et al. 2005). Moreover, all processes of ribosome biogenesis in cells proceed smoothly under low concentrations of cations such as Mg2+ and K+, in contrast to the conventional assembly experiments in which aberrantly high concentrations of cations are required.

Two studies on in vitro 30S subunit reconstitution revealed that activation of the RI can be achieved without heat activation. Culver and Noller (1999) demonstrated

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that recombinant 30S ribosomal proteins, which were individually expressed in *E. coli* cells, can be used for 30S subunit reconstitution. They also succeeded in activating the RI in the presence of protein chaperones instead of heat treatment (Maki et al. 2002). Jewett et al. (2013) developed the iSAT system, which allowed one-step coactivation of rRNA transcription and ribosome assembly in the same compartment. They revealed that functional ribosomal subunits can be assembled under physiological conditions using the S150 fraction of cell extracts. These studies suggested that the conventional heat activation for ribosome assembly could be replaced by the enzymatic factor activity in the in vitro ribosome reconstitution system.

In cells, several factors are known to be involved in the biosynthesis of 30S subunits (Shajani et al. 2011). In addition to the enzymes responsible for the post-transcriptional modification of rRNA and post-translational modification of ribosomal proteins, GTPase proteins, RNA helicases and other proteins have been suggested by various studies to participate in the assembly process in vivo that occurs cotranscriptionally. However, few studies in which the in vitro assembly experiments of 30S subunits were performed using these factors have been reported (Thurlow et al. 2016). Because some in vitro studies indicated that these factors effectively promote the rapid binding of ribosomal proteins to 16S rRNA (Bunner et al. 2010; Thurlow et al. 2016), it is necessary to construct a system using defined biogenesis factors under physiological conditions. By developing such a system, it could be possible to examine efficient 30S subunit reconstitution in a cell-like state with defined factors.

For this aim, we examined in vitro 30S subunit reconstitution using recombinantly expressed ribosomal proteins and biogenesis factors (Supplemental Table 1). First, S2–S21 ribosomal proteins possessing the same sequences as native ribosomal proteins were expressed as His-tagged SUMO fusion proteins (Malakhov et al. 2004). The His-tagged SUMO components were removed by the active domain of Ulp1 protease (Ulp1p), which cleaves peptide bonds after the SUMO coding end (Li and Hochstrasser 1999). By placing the N-terminal sequence of the ribosomal protein directly downstream from the SUMO coding end, ribosomal proteins were designed to be purified in the same forms as the native ribosomal proteins. Ulp1p cannot cleave sites between the SUMO coding end and a proline residue. Because S7, S19, and S21 retained a proline residue at their N-termini, the proline residues of these proteins were replaced by alanine residues for efficient cleavage. All SUMO fusion ribosomal proteins were overexpressed and purified by Ni2+ column chromatography, digested by Ulp1p, and then again subjected to Ni2+ column chromatography to remove SUMO components and His-tagged Ulp1p. In cases in which the ribosomal proteins were insoluble, which is consistent with previous results (Culver and Noller 1999), we used urea to solubilize the proteins (Supplemental Information).

The purity of prepared ribosomal proteins was analyzed by SDS-PAGE (Fig. 1), and their molecular weights were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

![FIGURE 1. S2–S21 30S subunit ribosomal protein after removal of the His tag and SUMO. Purified S2–S21 30S subunit ribosomal proteins after two-step chromatography (Supplemental Information) were subjected to 15% SDS-PAGE.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6182527/bin/Supplemental_Diagram.png)
(Supplemental Table 2). All mass spectra of S2–S21 30S subunit ribosomal proteins were measured as described by Talkington et al. (2005). In our study, mass spectra were calibrated using native ribosomal proteins. As a result, all purified recombinant ribosomal proteins, except S11 and S20, were confirmed with the correct molecular weights by MALDI-TOF-MS. The estimation of molecular weight indicated that S5 and S18 were not acetylated and that S12 was not methylthiolated, which differed from the native forms.

Reconstitution of 30S subunit components under high-salt conditions

We next examined a conventional 30S subunit reconstitution method using purified ribosomal proteins. We followed the method described by Culver and Noller (1999), which requires heat activation of the RI at 42°C under high-salt conditions. The resultant reconstituted 30S subunits exhibited similar sedimentation to the native 30S subunits in SDG analysis (Fig. 2A). The 30S fractions were collected and purified for analysis by SDS-PAGE. The reconstituted 30S subunits contained all S2–S21 ribosomal proteins (Fig. 2B), indicating that reconstitution of 30S subunits containing all 30S subunit proteins was successful using purified components. The band intensity of S2 of the reconstituted 30S was weaker than that in native 30S, suggesting heterogeneity in the stoichiometry of ribosomal proteins in the reconstituted 30S subunits.

Evaluation of the activity of the reconstituted 30S subunits

The poly(U)-directed polyphenylalanine synthesis activity of the reconstituted 30S subunits was evaluated. The activity of the reconstituted 30S subunits was ~30% of that of the native 30S subunits (Fig. 2C). We also tested the effect of adding S1 ribosomal protein on the activity of the reconstituted 30S subunit (Supplemental Fig. 1). Addition of the S1 protein to the reconstituted 30S subunits increased their activity by more than twofold, consistent with previous studies reporting that the polyphenylalanine synthesis activity of S1-depleted E. coli 30S subunits is enhanced by the addition of S1 (Roberts and Rabinowitz 1989). The entire translation activity of the reconstituted 30S subunits was further analyzed on the basis of DHFR synthesis in the PURE system (Fig. 2D), which demonstrated that the reconstituted subunits could synthesize DHFR and that this activity was increased upon adding S1. The reconstituted 30S subunits with S1 protein exhibited ~30% of the activity of native 30S subunits, whereas those without S1 protein were <10% of the activity of native 30S subunits.

FIGURE 2. Analysis of 30S particles reconstituted using conventional methods. (A) Reconstituted mixtures were analyzed by 10%–40% SDG analysis. Native 16S rRNA and native 30S subunits were analyzed as controls. The lines show the peaks of native 16S rRNA, native 30S subunits, and reconstituted 30S particles, using heat activation. (B) Purified reconstituted particles were analyzed by 15% SDS-PAGE and stained by SYPRO Orange (Invitrogen). Native 30S subunit proteins were loaded in the left lane, and 30S particles reconstituted via heat activation were loaded in the right lane. (C) Synthesized polyphenylalanine labeled with 14C was detected using a liquid scintillation counter. The values of 14C-phenylalanine incorporation by various subunits is denoted as follows: native 70S, ○; native 30S and 50S subunits, ▲; reconstituted 30S subunits and native 50S subunits, △; native 50S subunits, ▪; and no ribosomes, ◆. The bar shows the standardized values. (D) Dihydrofolate reductase (DHFR) synthesis in the PURE system using 30S subunits reconstituted via heat activation.
**PURE direct assay: in vitro reconstitution of 30S subunits with biogenesis factors**

In addition to the conventional method, we tried to identify more physiological ribosome reconstitution conditions without heat activation or high-salt conditions, such as the iSAT system, which enabled the integration of rRNA transcription and ribosome assembly (Jewett et al. 2013). For this purpose, we attempted to integrate ribosome assembly and the subsequent translation reaction by the assembled 30S subunits in a single tube using a buffer condition similar to that of the PURE system (Shimizu et al. 2005). We defined 200 mM K⁺ and 7.5 mM Mg²⁺ as the middle-salt condition and 150 mM K⁺ and 5 mM Mg²⁺ as the low-salt condition. The Mg²⁺ concentration was verified to not inhibit poly(U)-directed polyphenylalanine synthesis (Supplemental Fig. 2).

We examined whether biogenesis factors facilitate 30S subunit assembly in the absence of heat activation. Eight enzymes or factors including modification enzymes were selected, purified (Supplemental Fig. 3), and evaluated in an assay system in which successive 30S subunit assembly and poly(U)-directed polyphenylalanine synthesis were performed in a test tube (PURE direct assay). In the absence of biogenesis factors, the addition of both 16S rRNA and ribosomal proteins did not affect ¹⁴C-phenylalanine incorporation under the low-salt condition, whereas its incorporation was increased in the middle-salt condition, suggesting 30S subunit assembly promoted by 16S rRNA and ribosomal proteins in the middle-salt condition (Fig. 3A). In contrast, in the presence of biogenesis factors, the addition of both 16S rRNA and ribosomal proteins increased the ¹⁴C-phenylalanine incorporation under both conditions, suggesting the biogenesis factors promoted the 30S subunit assembly even under low-salt conditions. We have no explanation for the high background, but it is likely that contamination of 30S subunits in the 50S fraction in PURE direct assay caused this high background.

The 30S subunit assembly in the low-salt condition was further verified via SDG analysis and two-step PURE direct assay (Fig. 3B). We divided the 30S subunit assembly reaction into two parts: an assembly reaction followed by a translation reaction. In the first part, the assembly reaction was performed for 40 min at 37°C without translation mixture, including components such as native 50S subunits, poly(U), ¹⁴C-phenylalanine and elongation factors. Subsequently, the assembly reaction was additionally performed for 20 min at 37°C (II—16S rRNA, II, III) or 42°C (IV) to analyze the effect of heat-treatment. After the assembly reaction, the level of polyphenylalanine synthesis was monitored at 30 and 60 min after the addition of the translation mixture.
assay. In the absence of biogenesis factors, a peak with smaller sedimentation compared to that of the native 30S subunits was observed (Fig. 3B, II), suggesting most of the formed particles are those that stopped developing during 30S subunit formation. When the biogenesis factors were added to the reconstitution mixtures, the peak for immature particles shifted to the 30S position (Fig. 3B, III), and when heat activation was performed in the presence of biogenesis factors, a sharp and large peak at the 30S position was observed (Fig. 3B, IV). The results indicate that biogenesis factors facilitate in vitro 30S subunit assembly. Activities of assembled 30S subunits, which were analyzed by the two-step PURE direct assay (in Fig. 3B), were nearly in proportion to the intensity of the peak at the 30S position in the SDG analysis. Together with the result of PURE direct assay, in vitro 30S subunit reconstitution can be performed using biogenesis factors under low-salt conditions with 150 mM K⁺ and 5 mM Mg²⁺.

The effects of ribosome biogenesis factors on in vitro 30S subunit reconstitution

The effect of individual biogenesis factors and DnaK-DnaJ-GrpE was examined using the PURE direct assay (Fig. 4A). In this experiment, Era displayed the strongest effect on 30S subunit assembly, whereas YjeQ and RimP slightly enhanced assembly. Era and YjeQ are responsible for the late stage of assembly. RimP was also identified as a biogenesis factor involved in the late stages of 30S subunit biogenesis (Bunner et al. 2010).

Previous reports indicated that two GTPases worked effectively in in vitro 30S subunit reconstitution (Thurlow et al. 2016) and that the GTPase activity of YjeQ is enhanced in the presence of mature 30S or 70S subunits, suggesting that these subunits may function as GTPase-activating factors (Daigle and Brown 2004). We observed the GTPase activity of YjeQ depending on the 30S or 70S subunits under the low-salt concentration (Supplemental Fig. 4), which is consistent with the previous report. In contrast, the increase of GTPase activity upon the addition of ribosomes was not observed for Era at 20 nM concentration. This is consistent with previous reports that 50 nM each of Era and subunits did not enhance GTPase activity (Thurlow et al. 2016). Additionally, we examined the time-dependent increase of 14C-phenylalanine incorporation in the PURE direct assay in the presence of Era, and it turned out that Era increased the efficiency of in vitro 30S subunit reconstitution under the low-salt condition (Fig. 4B).

DISCUSSION

In this study, ribosomal proteins were prepared separately by expressing the proteins in their SUMO fusion forms followed by Ulp1 protein digestion. The fusion with SUMO has advantages to prevent recombinant ribosomal proteins of aggregation and of degradation during the purification procedure. In addition, the removal of SUMO with Ulp1 can generate protein with native amino acid sequence, which is different from the digestion by TEV protease or thrombin. The amino acid sequence of recombinant ribosomal proteins after cleavage is expected to be identical to that of the native ribosomal proteins, including N-terminal methionine excision (Arnold and Reilly 1999), except when proline is located at the C-terminal end of the Ulp1 cleavage site. As S7, S19, and S21 have proline residues at their N-termini, proline was replaced by alanine in these proteins. As N-terminal acetylation is not introduced in this method, the influence of these post-translational modifications on the process of reconstitution and the effect on ribosome activity can be evaluated, which differs from...
Culver’s work, in which they believed that N-terminal acet-
ylation might be substoichiometrically introduced (Culver
and Noller 1999). The purification procedure using the af-
finity tag is advantageous due to the uniform preparation
procedure regardless of the properties of the proteins.
However, most ribosomal proteins are inclined to aggre-
gate during the purification procedures from overex-
pressed cells as reported by Culver and Noller (1999).
This propensity of ribosomal proteins is considered to be
caused by the exposure of positively charged amino acids
on the surface to rRNA. In particular, S9, S10, S11, and S18
had high insolubility, and they were stored in a urea-con-
taining solution. To purify such proteins in a solubilized
state, it is necessary to include steps such as supplying part-
nner nucleic acids as decoys or coupling RNA transcription
with ribosome assembly as done in the ISAT system.

Using recombinant ribosomal proteins, 3OS reconsti-
tution was performed in a conventional manner under a
high-salt condition. The reconstituted particles displayed
a similar sedimentation pattern as native 3OS. The addition
of S1 protein to reconstituted particles enhanced the level
of polyphenylalanine synthesis to ~80% of that of native
3OS (Supplemental Fig. 1). Roberts and Rabinowitz report-
eted that poly(U)-directed polyphenylalanine synthesis was
activated in E. coli 3OS subunits lacking S1 by the addition
of the protein (Roberts and Rabinowitz 1989). In Culver’s
report, whereas the translational activity of ribosomes re-
constituted from TP30 proteins was 79% of that of native
ribosomes, those reconstituted from recombinant proteins
exhibited 34% of the activity of native ribosomes. Because
they did not use S1 protein in their reconstitution experi-
ment from recombinant proteins, the discrepancy might be
caused by the presence or absence of S1 protein, which is
consistent with our present observation (Fig. 2C).

In contrast to poly(U)-directed translation, the efficiency
of DHFR synthesis by the reconstituted subunits was rela-
tively low compared to that of native 3OS (Fig. 2D). The
pattern of ribosomal proteins in SDS-PAGE for the ob-
tained 3OS particles could explain the low productivity
(Fig. 2B). Specifically, the band corresponding to S2 was
thin, and the proportion of fully reconstituted 30S was
low. According to Nomura’s 30S subunit assembly map,
S2 binds 16S rRNA in the final step, indicating that our re-
constituted 30S subunits are probably composed of vari-
ous immature complexes that stopped developing
before S2 binding. Although SDS-PAGE analysis failed to
detect the distinct depletion of particular ribosomal pro-
teins, it is plausible that the reconstituted 30S sample con-
tains heterogeneous immature particles.

Recently, heterogeneity of immature particles formed in
mutant cells was demonstrated using quantitative mass
spectrometry and single-particle cryo-electron microscopy
(Davis et al. 2016). The methods are also applicable to the
in vitro reconstituted particles and may provide insight into
the ribosome assembly process for efficient reconstitution
in vitro. Analyses of in vitro reconstituted particles using
these methods are expected to be useful, because inter-
mediate particles of early stages that are difficult to
isolate from cells can be prepared. The combination of
these analyses and examination of translational activities
of reconstituted subunits will allow us to elucidate function
of biogenesis factors and to optimize assembly conditions,
such as biogenesis factors, ion-strength, and temperature.

To investigate the activities of 30S subunit biogenesis
factors, we developed the PURE direct assay system, which
allowed us to evaluate their effects on in vitro 30S subunit
assembly. We found that Era and YjeQ have distinct effects
on in vitro 30S subunit assembly (Fig. 4A). Both GTPases
were identified decades ago. Early genetic studies sug-
gested that Era and YjeQ were involved in 30S subunit bi-
genesis because their mutant phenotype often exhibit 30S
subunit assembly defects. Later biochemical experiments
revealed that their GTPase activities are activated by 16S
rRNA and 30S subunits (Sharma et al. 2005). Cryo-electron
microscopy images suggested that Era binds to the 16S
rRNA 3′-terminal region where S1 protein binds (Sharma
et al. 2005). Tu et al. (2009) suggested Era serves as a chap-
erone for processing, maturation of 16S rRNA and a check-
point for assembly of the 30S ribosomal subunit. Our ex-
perimental data also showed that Era can work before S1
binding. In this study, we observed that Era promotes
30S subunit assembly in vitro by associating with mature
16S rRNA possessing modified nucleotides. Furthermore,
this experiment was performed using a half-molar ratio of
30S subunit biogenesis factors to 16S rRNA, which is a
much higher concentration than that in cells. Under these
conditions, multiple pathways for completing 30S subunit
assembly were selected and passed by RIs, which take the
easiest path in 30S subunit assembly landscapes. In vitro
30S subunit assembly using purified components, under-
modified ribosomal proteins and mature 16S rRNA is high-
ly accelerated by Era and RimP, and these two genes could
not be replaced by any other factors. These findings are
consistent with previous observations that RimP could
not be rescued by any other biogenesis factor (Nord
et al. 2009) and that Era remains bound to the immature
30S subunit throughout several stages of assembly with
other biogenesis factors (Mulder et al. 2010).

The achievement of de novo synthesis of ribosomes us-
ing cell-free translation is desired in the field of synthetic
biology. In addition, it would facilitate the development
of an artificial evolution system of functional ribosomes
and permit the bottom-up synthesis of minimal cells. At
present, it is possible to reconstitute functional 30S sub-
units from TP30 and cotranscribed 16S RNA without mod-
ification under physiological conditions, although the
efficiency of the subunits is lower than that when native
16S rRNA is used (Jewett et al. 2013). Unlike 30S subunits,
the reconstitution of catalytically active 50S subunits using
in vitro transcribed 23S RNA under physiological

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conditions has not been realized. These results indicate that modifications within native rRNA are important for reconstructing ribosomes efficiently under physiological conditions as well as increasing translation efficiency and fidelity. Therefore, it will be necessary to develop an in vitro system for incorporating modified nucleotides into transcribed rRNAs or perform the selection of highly active 23S rRNA mutants via artificial evolution. Moreover, for the de novo synthesis of ribosomes, the expression of more than 50 types of ribosomal proteins and rRNA transcripts must be combined simultaneously. Church demonstrated that individual ribosomal proteins can be expressed in cell-free translation systems and that 30S subunits can be reconstituted using ribosomal proteins to prepare using cell-free translation systems (data not shown). These studies will facilitate the de novo synthesis of ribosomes.

MATERIALS AND METHODS

Protein purification procedure

Ribosomal proteins were expressed in E. coli cells in their His-tagged SUMO fusion forms and purified using Ni-column chromatography. The SUMO portion was removed via Ulp1 digestion, and ribosomal proteins were further purified using column chromatography. Details of the procedure are described in the Supplemental Information.

Preparation of native components in ribosomes

Tightly coupled ribosomes were purified from E. coli A19 cells via sucrose density gradient (SDG) centrifugation as previously described (Spedding 1999). 70S ribosomes were diluted in SE buffer [20 mM Hepes-KOH (pH 7.6), 30 mM NH4Cl, 1 mM Mg(OAc)2, 7 mM ß-mercaptoethanol]. Diluted 70S ribosomes were loaded onto 10%–40% sucrose gradients in SE buffer and ultracentrifuged in an SW28 rotor at 18,000 rpm (Beckman Coulter) for 16 h at 4°C. The fractions exhibiting similar sedimentation distances as 30S and 50S subunits were collected by a piston gradient fractionator (BioComp Instruments, Inc.) and subjected to further ultracentrifugation in a 45 Ti rotor at 40,000 rpm (Beckman Coulter) for 36 h at 4°C for recovery. 30S and 50S subunits were resolved in ribosome buffer (20 mM Hepes-KOH [pH 7.6], 30 mM KCl, 6 mM Mg(OAc)2, 7 mM ß-mercaptoethanol). Aliquots of the subunits were frozen at −80°C. Native 16S rRNA was extracted from 30S subunits using phenol and chloroform in the presence of 270 mM KOAc (pH 5.2). RNA was precipitated by the addition of 0.9 volumes of 2-propanol and collected by centrifugation. Pellets were washed with 80% ethanol, and supernatants were removed after centrifugation, dried under vacuum, and resuspended in Milli-Q water. The concentration of native 16S rRNA was measured using Gene Spec I (HITACHI). Aliquots of the RNA samples were frozen at −80°C.

Reconstitution of 30S subunits by conventional procedure

Forty picomoles of native 16S rRNA extracted from 30S subunits were incubated for 15 min at 42°C followed by 20 min on ice. Group I proteins containing 160 pmol each of S4, S7, S8, S15, S17, and S20 were added to native 16S rRNA with 40 units of human placenta RNase inhibitor (HPRI, Takara). Buffer conditions were adjusted to R buffer (50 mM Hepes-KOH [pH 7.6], 330 mM KCl, 20 mM MgCl2, 7 mM ß-mercaptoethanol) and reaction mixtures were incubated for 20 min at 37°C. Group II proteins containing 160 pmol each of S5, S6, S9, S11, S12, S13, S16, S18, and S19 were added to reaction mixtures with 40 units of HPRI. Buffer conditions were adjusted to R buffer, and reaction mixtures were incubated for 20 min at 37°C followed by 1 h at 42°C. Group III proteins containing 160 pmol each of S2, S3, S10, S14, and S21 were added to reaction mixtures with 40 units of HPRI. Buffer conditions were adjusted to R buffer, and reaction mixtures were incubated for 20 min at 37°C. The final concentrations of native 16S rRNA and S2–S21 ribosomal proteins were 0.4 and 1.6 μM, respectively, in 100 μL. Reconstitution of 30S subunits was analyzed by sucrose gradient sedimentation using 10%–40% sucrose gradients in 20 mM Hepes-KOH (pH 7.6), 20 mM MgCl2, and 330 mM KCl in a SW41Ti rotor at 32,000 rpm (Beckman Coulter) for 15.5 h at 4°C.

Purification of reconstituted 30S subunits

Fractions with similar sedimentation velocity in sucrose gradients as native 30S subunits were collected and diluted with R buffer, followed by ultracentrifugation using a TLA100.3 rotor (Beckman Coulter) at 70,000 rpm for 16 h at 4°C for recovery. Reconstituted 30S subunits were resolved in ribosome buffer, and the concentration was measured using Gene Spec I (HITACHI). Aliquots of the reconstituted subunits were frozen at −80°C.

Measurement of the peptide or protein synthesis activity of reconstituted 30S subunits

The final concentrations of components used in the reaction mixtures were usually 50 mM Hepes-KOH (pH 7.6), 100 mM potassium glutamate, 5–20 mM Mg(OAc)2, 2 mM spermidine, 1 mM DTT, 1 mM ATP, 1 mM GTP, 0.5 pmol of isolated 30S subunits, 0.5 pmol of native 50S subunits, 56 A260 units of tRNA mix (Roche), 10 μM 13C-phenylalanine, 800 ng of poly(U), 1 μg of EF-G, 2 μg of EF-Tu, 1 μg of EF-Ts, 0.33 μg of PhoRS, and 16 pmol of S1. The total volume of the reaction mixture was 20 μL. The reaction mixture was incubated at 37°C. Reactions were terminated at 60 min by spotting onto filter paper (Whatman 3MM), and the filters were submerged in ice-cold 10% TCA. After boiling in 10% TCA, the filters were washed twice with 10% TCA and 100% ethanol and dried. Radioactivity was measured using a liquid scintillation counter. Protein synthesis using the PURE system was performed as described by Shimizu et al. (2005). Reaction mixtures containing 2.4 pmol of 30S subunits, 2.4 pmol of native 50S subunits, 1 μg of S1, 35S-methionine and other required components were incubated for 60 min at 37°C. Synthesized proteins labeled with 35S-methionine were analyzed by 15% SDS-PAGE, and the radioactivity of the product was measured.
PURE direct assay

The final concentrations of components used in the reaction mixtures were 50 mM Hepes-KOH (pH 7.6), 150–200 mM potassium glutamate, 5–7.5 mM MgCl₂, 2 mM spermidine, 1 mM DTT, 50 µM acetyl coenzyme A, 1 mM ATP, 1 mM GTP, 0.5 pmol of native 30S subunits, 56 A₂₆₀ units of tRNA mix (Roche), 10 µM ¹⁴C-phenylalanine, 20 µg of poly(U), 1 µg of EF-G, 2 µg of EF-Tu, 1 µg of EF-Ts, 0.33 µg of PheRS, 8 pmol of S1, 24 units of HPRI (Takara), 4 pmol of native 16S rRNA, 16 pmol each of S2–S21 ribosomal proteins, and 4 pmol of each biogenesis factor. The components were incubated in a reaction volume of 20 µL for 60 min at 37°C. Reactions were stopped via spotting onto filter paper (Whatman 3MM) after 60 min, and filters were submerged in ice-cold 10% TCA after spotting. Filters were washed three times with 10% TCA and 100% ethanol, dried, and counted.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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