Possible involvement of *Escherichia coli* 23S ribosomal RNA in peptide bond formation

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ABSTRACT
Experimental results are presented suggesting that 23S rRNA is directly involved in the peptide bond formation usually performed on the ribosome. Although several reports have indicated that the eubacterial peptidyltransferase reaction does not necessarily require all the ribosomal proteins, the reconstitution of peptidyltransferase activity by a naked 23S rRNA without the help of any of the ribosomal proteins has not been reported previously. It is demonstrated that an *E. coli* 23S rRNA transcript synthesized by T7 RNA polymerase in vitro was able to promote peptide bond formation in the presence of 0.5% SDS. The reaction was inhibited by the peptidyltransferase-specific antibiotics chloramphenicol and carbomycin, and by digestion with RNases A and T1. Site-directed mutageneses at two highly conserved regions close to the peptidyltransferase center ring, G2252 to U2252 and C2507G2581 to U2507A2581, also suppressed peptide bond formation. These findings strongly suggest that 23S rRNA is the peptidyltransferase itself.

Keywords: carbomycin; chloramphenicol; N-acetylphenylalanyl-tRNA<sup>Phe</sup>; peptidyltransferase; phenylalanyl-tRNA<sup>Phe</sup>; site-directed mutation

INTRODUCTION
Nucleic acids are synthesized with the help of proteins, and proteins are synthesized under the control of their corresponding nucleotide sequences. One of the tasks of molecular biology is to elucidate the molecular mechanism involved in the functioning of this interdependent system. A promising approach lies in investigating the ribosome, because the ribosome, itself composed of nucleic acids and proteins, plays the central role in protein synthesis in the living cell, in which the genetic information of nucleic acids is converted into proteins.

A milestone on the way toward achieving an understanding of the mechanism was reached with the discovery of catalytic RNA (Cech et al., 1981; Guerrier-Tanaka et al., 1983), which led to the fascinating concept that RNA enzymes had worked prior to the emergence of protein enzymes (Waldrop, 1992). Even at the present time, RNA molecules—mRNA, tRNA, and rRNA—have significant functions in translation, and some recent reports have suggested the direct involvement of eubacterial 23S rRNA in peptide bond formation on the ribosomal large subunit (reviewed in Noller, 1991).

Schulze and Nierhaus (1982) reported that the peptidyltransferase activity of the *Escherichia coli* ribosomal large subunit requires only a few of its component proteins—L2, L3, L4, L15, and L16—in addition to 23S rRNA. It has also been shown that L15 and L16 are merely assembly proteins, which accelerate the formation of active ribosomes during the late assembly step, but are rather dispensable for peptidyltransferase activity (Franceschi & Nierhaus, 1990). Noller and his co-workers have demonstrated that the peptidyltransferase activities of *E. coli* and *Thermus aquaticus* ribosomes are resistant to conventional protein extraction procedures (Noller et al., 1992), and that the formation of a G-C pair between G2252 in the conserved hairpin loop of *E. coli* 23S rRNA and C74 in the 3′-terminal region of the tRNA is a prerequisite for the activity (Samaha et al., 1995). These findings thus suggest that not all the large subunit proteins are necessarily required for peptidyltransferase activity.

The peptidyltransferase reaction is typical of the nucleophilic substitution reactions that occur in biological metabolism. In organic synthesis, pyridine is known to be a useful solvent for this kind of reaction. Taking advantage of this, by utilizing ribosomes isolated from
various kinds of organisms, we found that, without soluble protein factors, a high concentration (50–60%) of pyridine is able to promote the synthetic polyn(U), polyn(A), and alternate polyn(UC)-dependent syntheses of polyn(Phe), polyn(Lys), and alternate copoly(Ser-Leu), respectively (Nitta et al., 1994, 1995; Nojima et al., 1996), and that, under these conditions, only 23S rRNA and two-thirds of the ribosomal proteins of the large subunit remain in the active E. coli ribosomal particle (unpubl. data). We also determined that, unlike conventional cell-free translation systems, this pyridine system is activated by SDS (Nitta et al., 1995).

Based on these findings, we constructed a novel assay system for the detection of peptidyltransferase activity that uses a naked 23S rRNA in the presence of only SDS. It was found that an E. coli 23S rRNA transcript without any ribosomal proteins was capable of synthesizing N-acetylphenylalanylphenylalanine (AcPhe-Phe) from N-acetylphenylalanyl-tRNA\textsuperscript{phe} (AcPhe-tRNA) and phenylalanyl-tRNA\textsuperscript{phe} (Phe-tRNA) of E. coli with the sole help of SDS.

**RESULTS**

Peptide bond formation by 23S rRNA transcripts

The 23S rRNA transcripts were prepared according to Ofengand (Weitzmann et al., 1990), with the slight modification that direct PCR amplification of the rRNA gene in the E. coli genome was employed. Amplification of the rRNA gene was confirmed by sequencing using the dideoxy method, from which 23S rRNA was successfully obtained by transcription in vitro with T7 RNA polymerase. After transcription, the 23S rRNA transcripts could be purified conventionally by gel filtration (usually with Sephadex G50) or by ion exchange chromatography with Qiagen (Qiagen Inc.). When Qiagen was used for purification, the transcript was eluted with a high concentration (6 M) of urea in order to remove template DNA and NTPs completely. Because rRNA transcripts should be free of urea and other contaminants for peptide bond formation activity, we have devised a special protocol (described below) in which purification with Qiagen is followed by dialysis against a self-folding buffer consisting of 50 mM $\text{HEPES-KOH}$, pH 7.5, 20 mM Mg(AcO)\textsubscript{2}, and 400 mM $\text{NH}_4\text{Cl}$.

Some researchers have reported that rRNAs, like proteins, are only capable of being self-folded into tertiary structures similar to those in the ribosomal subunits (reviewed in Vasiliev et al., 1986) if they are treated under appropriate conditions, i.e., in a buffer that contains only Mg\textsuperscript{2+}, spermidine, or ethanol with Mg\textsuperscript{2+}. From these three alternatives, we chose as our self-folding buffer one containing only Mg\textsuperscript{2+}; although ethanol was capable of activating the peptidyltransferase reaction, it is also possibly used for the synthesis of a side product, phenylalanine methyl ester, whereas spermidine is one of the polynamines that have no enhancing activity (data not shown). The self-folding buffer was the same as that used in the second-step of the in vitro reconstitution of the total 50S subunit and 70S ribosome except that EDTA and $\beta$-mercaptoethanol were omitted (reviewed in Nierhaus, 1990). After elution of the 23S rRNA transcript from the Qiagen resin, it was further dialyzed against the self-folding buffer to remove urea, which allowed effective self-folding of the transcript (see below).

Refolding of the 23S rRNA transcript was a prerequisite for peptide bond formation activity. A denatured 23S rRNA transcript eluted just by 6 M urea had no activity at all (data not shown). After dialysis against the self-folding buffer, the viscosity of the solution containing the rRNA transcripts decreased considerably as could be judged subjectively during pipetting. In parallel with this phenomenon, peptidyltransferase activity emerged (Fig. 1A, lane 1). Lanes 2 and 3 in Figure 1A also indicate the importance of the active conformation of 23S rRNA for peptidyltransferase activity: after 23S rRNA transcripts were denatured by heating at 95°C for 5 min, they were cooled either gradually (lane 2) or immediately (lane 3). The rRNA treated by the gradual cooling procedure had peptidyltransferase activity, suggesting that it could be refolded during cooling, whereas the rRNA treated by rapid quenching was inactive, indicating that it remained in the denatured state. The denatured transcript could be re-activated by heating at 50°C for 30 min, which is a similar procedure to that used for the in vitro reconstitution of the total ribosome (data not shown) (Nierhaus, 1990). The results in Figure 1A, lanes 4 and 5, clearly indicate that the peptide bond formation reaction requires both 23S rRNA and SDS.

In the presence of only AcPhe-tRNA as a substrate, no product was formed other than AcPhe and an unknown byproduct (Fig. 1A, lane 7). In the presence of only Phe-tRNA, no spot corresponding to AcPhe-Phe could be observed, but a faint spot corresponding to phenylalanylphenylalanine (Phe-Phe) was detected (Fig. 1A, lane 8). The yield of the product resulting from peptide bond formation was calculated as the amount of the radioactivity of the spot corresponding to the product divided by the total radioactivity of all the spots in the relevant lane. Thus, AcPhe-Phe (lane 1) and Phe-Phe (lane 8) were estimated to be formed by 1.8 and 0.12% of the input radioactivity, respectively; the yield of Phe-Phe, even in the absence of AcPhe-tRNA as a substrate, was less than 1/10 that of AcPhe-Phe. This result suggests that, in the active rRNA fraction at least, the conformation of the self-folded 23S rRNA was close to that in the total ribosome, and that the naked 23S rRNA was probably bound by tRNAs in a...
Peptide bond formation by 23S rRNA

FIGURE 1. Involvement of 23S rRNA transcripts in peptide bond formation. A: E. coli 23S rRNA prepared by T7 RNA polymerase was dialyzed against a self-folding buffer: 50 mM HEPES-KOH, pH 7.5, 20 mM Mg(OAc)$_2$, and 400 mM NH$_4$Cl (indicated by F, folding). A portion of the reaction mixture was heated successively at 95 °C for 5 min and then cooled to 25 °C over a period of 70 min (indicated by R, re-folding) or quenched on ice (indicated by D, deratturing). AcPhe-Phe synthesis was performed at 37 °C for 2 h in 25 μL of a reaction mixture containing 1.1 pmol/μL Ac$_{[14}$C]Phe-tRNA$^{\text{Phe}}$ from E. coli (specific activity: 9.2 × 10$^{-4}$ pmol/cpm), 7.2 pmol/μL 23S rRNA, 50 mM HEPES-KOH, pH 8.2, 100 mM MgCl$_2$, and 0.5% SDS. After incubation, the reaction was stopped and the aminoacyl bond was hydrolyzed by heating for 15 min at 95 °C. The products contained in the supernatant (20 μL) of a reaction mixture containing 1 pmol LAc$_{[14}$C]Phe-tRNA$^{\text{Phe}}$ and 2 pmol AcPhe-tRNA$^{\text{Phe}}$ synthesized in the reaction mixture were analyzed by TLC [silica gel plate (Merck) developed with nBuOH/AC/$_2$O/H$_2$O = 4/0.9/1]. All the procedures were performed at above room temperature to avoid precipitation of the products with SDS. Phe, AcPhe, AcPhe-Phe, and Phe-Phe stand for phenylalanine, N-acetylphenylalanine, N-acetylphenylalanylphenylalanine, and phenylalanylphenylalanine, respectively. Lanes 4–8 show the control experiments without 23S rRNA, SDS, both 23S rRNA and SDS, Phe-tRNA, and AcPhe-tRNA, respectively. Asterisks denote unknown side products. B: Effect of reaction conditions on AcPhe-Phe synthesis. The reaction was performed under various conditions and the yields of AcPhe-Phe were calculated by dividing the radioactivity of the spot corresponding to AcPhe-Phe by the sum of the radioactivities of all the spots in the relevant lane on the TLC plate. A yield of 1% in AcPhe-Phe formation corresponds to 1.5 peptide bonds formed by 1,000 pieces of 23S rRNA. STD (standard condition) means that the reaction was performed at 37 °C in the above reaction mixture containing 100 mM MgCl$_2$ and 0.5% SDS; – SDS, without SDS; – Mg$^{2+}$, complete chelation of Mg$^{2+}$ in the reaction mixture by addition of excess (250 mM) EDTA; (ND means that no AcPhe-Phe was detected); 20 mM Mg$^{2+}$, the final concentration of Mg$^{2+}$ was decreased to 20 mM. The reaction was performed with the same concentration of Mg$^{2+}$ and SDS as in the STD at pHs 7.7, 8.0, and 9.2, and at incubation temperatures of 30 and 45 °C, as indicated. Assays were performed 3–5 times and the mean values are shown with the experimental error. C: Time course of the AcPhe-Phe formation reaction at 30 °C (×), 37 °C (●), and 45 °C (○). D: Effect of AcPhe- and Phe-tRNA concentrations on the AcPhe-Phe formation reaction in 30-min incubation at high (7.2 pmol/μL, ○), medium (2.4 pmol/μL, ●), and low (0.9 pmol/μL, ×) concentrations of 23S rRNA. The concentration of both tRNAs was the same in each reaction mixture.

similar manner to that in the total ribosome so as to produce peptide bonds.

The conventional puromycin reaction (reviewed in Monro, 1971), in which an N-acetylamino acid moiety at the 3’-terminal of tRNA at the P site is transferred to puromycin at the A site on the ribosomal large subunit in the presence of 33% methanol or ethanol, has been used as an assay for evaluating peptidyltransferase activity. In our system, however, this reaction did not work (see Discussion). It is thus presumed that the
proper tRNA binding sites corresponding to the usual P and A sites on the ribosome are arranged even on 23S rRNA, where they are occupied by AcPhe-tRNA and Phe-tRNA, respectively, and hence AcPhe-Phe is formed with the peptidyltransferase activity exerted by the 23S rRNA (Fig. 1A).

Figure 1B shows that AcPhe-Phe formation was enhanced around 10-fold with 0.5% SDS. We found no enhancement by Triton X-100, a nonionic detergent. SDS might play a role in stabilizing the active conformation of 23S rRNA, in which the two aminoacyl-tRNAs are arranged in their proper positions. X-ray crystallography of biomolecules shows that surface active agents sometimes induce crystal growth of proteins and nucleic acids, whereas the activity of some RNA enzymes is known to be enhanced by SDS (Hosaka et al., 1994) and Triton X-100 (van Tol et al., 1989). Figure 1B also shows that AcPhe-Phe synthesis requires a high concentration of Mg2+ (100 mM), a relatively high pH, 8.2, and an incubation temperature close to that of the living cells (37°C). EDTA completely inhibited the activity, which may be due to Mg2+ depletion resulting in the conformational denaturation of the RNAs. The requirement of a high Mg2+ concentration is similar to that for the cleavage reaction of precursor tRNA by M1 RNA, the RNA component in RNase P. Although RNase P holoenzyme functions in 10 mM MgCl2, M1 RNA alone requires 100 mM MgCl2 for its cleavage activity (reviewed in Baer et al., 1990). The highest optimum pH for the in vitro translation catalyzed by ribosomes is normally 8.8–9.0, but, in our system, peptide bond formation activity almost disappeared at pH 9.2. One reason for the narrow pH range might be that, instead of the total ribosome, we used naked rRNA, which is more unstable at a relatively high pH. The low pH of the stock solution for Phe- and AcPhe-tRNA was adjusted with 1 N KOH, to the final pH of the reaction mixture for AcPhe-Phe synthesis.

Kinetic observations

If we view 23S rRNA as an enzyme and both AcPhe- and Phe-tRNAs as the substrates in this new assay system, the enzymology concerning 23S rRNA can be established by referring to the early work on group I introns (Herschlag & Cech, 1990). Because AcPhe-Phe barely became detectable during the initial 30-min incubation (Fig. 1C), we were obliged to estimate the initial velocity from the yield of AcPhe-Phe at 30 min. Figure 1D shows how the concentrations of AcPhe-tRNA and Phe-tRNA influenced the yields of AcPhe-Phe after 30-min incubation when the concentration of 23S rRNA was fixed at various levels. At relatively low concentrations of AcPhe- and Phe-tRNAs, the yields increased almost linearly with increments in the concentrations of both tRNAs, and leveled off at around 1–1.5 pmol/μL. These features indicate the stoichiometric binding of the AcPhe- and Phe-tRNAs to 23S rRNA.

We attempted to obtain some kinetic parameters of peptide bond formation from the data in Figure 1D; however, the system hardly obeyed Michaelis–Menten-type kinetics. The likely reason is that almost half the amount of Phe-tRNA was deacylated in 17-min incubation (see also Discussion). Thus, we are only able to surmise from the data in Figure 1D that the kcat might be on the order of 0.01 min−1. Although this is much smaller than the values found in poly(U)-dependent polyphenylalanine synthesis, which are comparable to the kcat observed in an in vivo system (ca. 100 min−1 as estimated from the work of Bartetzko & Nierhaus, 1988) and in the first-step of the endonuclease reaction (at 50°C) in the self-splicing of Tetrahymena pre-rRNA (0.1 min−1) (Herschlag & Cech, 1990), it may be improved by revision of the assay system, for example, by using 23S rRNA pre-saturated with AcPhe-tRNA as a starting complex to avoid nonspecific deacylation (see Discussion).

Identification of products

The reaction products were identified by TLC with two different solvent systems as well as by HPLC (Fig. 2). The chemical structures of AcPhe-Phe, Phe-Phe, and AcPhe were confirmed by two-dimensional NMR spectroscopy, including detection of the peptide bonds in AcPhe-Phe and Phe-Phe (data not shown). By comparing the Rf values of radioactive products after peptide bond formation with those of authentic samples, the spots were identified by the two kinds of TLC (Fig. 2A,B); two unidentified byproducts were detected, which may correspond to two of the peaks in the HPLC profile (Fig. 2C). The upper panel of Figure 2C shows the elution profile of the [14C]-labeled products (solid line) obtained in the presence of self-folded 23S rRNA, together with the authentic Phe, AcPhe, and AcPhe-Phe, which were coinjected and monitored by UV at 254 nm (broken line). Thus, the peak eluted at around 10 min was clearly identified as AcPhe-Phe. The lower panel shows the elution profile of the products obtained in the presence of denatured 23S rRNA. In this case, only a background level peak was observed at the retention time of AcPhe-Phe; however, both of the unknown byproducts still appeared in considerable amounts. In each analysis, the yields of AcPhe-Phe with self-folded and denatured 23S rRNAs were 1.5–1.9 and 0.1–0.3%, respectively. The formation of AcPhe-Phe was also confirmed by paper electrophoresis (Noller et al., 1992) (data not shown).

Sensitivity toward antibiotics, RNase digestion, and mutation

To verify that the activity of the naked 23S rRNA was actually due to peptidyltransferase, the effects on AcPhe-
Phe synthesis of chloramphenicol and carbomycin, both of which are inhibitors of prokaryote-specific peptidyltransferase (Moazed & Noller, 1987; Cundliffe, 1990), were examined. As shown in Figure 3A, both antibiotics inhibited the AcPhe-Phe formation reaction by ca. 70%; the yields of AcPhe-Phe in the absence of either inhibitor (lane 1), and in the presence of chloramphenicol (lane 2) or carbomycin (lane 3) were 1.5–1.9, 0.4–0.5, and 0.5–0.6%, respectively. Chloramphenicol was thus shown to be slightly more inhibitory than carbomycin.

Figure 3B shows that the AcPhe-Phe synthesis reaction could be eliminated completely by digestion of 23S rRNA with either pyrimidine-specific RNase A or guanine-specific RNase T1. These results are in agreement with the findings of earlier work on the E. coli intact ribosome (Cerna et al., 1973) and of a more recent report (Noller et al., 1992), in which RNase T1 digestion was used.

Phylogenetic comparison of the rRNA secondary structure clearly reveals that RNAs have been conserved to a remarkable degree across the whole of the evolutionary spectrum (reviewed in Raúe et al., 1988). G2252 of E. coli 23S rRNA is one of the nucleotides universally conserved at a helix near the so-called “pep-
tidyltransferase ring" of domain V (Noller, 1991). This nucleotide was protected from ketoxal treatment by the binding of tRNA and/or CCA-containing tRNA analogues to the P-site in the total ribosome (Moazed & Noller, 1989). Mutation at G2252 was dominantly lethal (Gregory et al., 1994). Further, a recent study has demonstrated that the base pair formation between G2252 and C74 in the 3' terminal region of tRNA is involved directly in peptidyltransferase activity (Samaha et al., 1995). Considering these results, site-directed mutagenesis of G2252 to U in 23S rRNA was tested for peptidyltransferase activity using our assay system. The results, shown in Figure 3C, demonstrate that a mutation of G2252 to U suppressed AcPhe-Phe formation by as much as ca. 60%, the yields of AcPhe-Phe with the wild-type and mutant 23S rRNAs being 1.8–2.0 and 0.6–0.8%, respectively.

On the other hand, Nierhaus and his coworkers have recently reported that site-directed mutageneses at G2581, and at other neighboring nucleotides in the secondary structure, such as C2507, severely impaired peptidyltransferase activity. They also found that thiostrepton, which blocks translocation but not peptidyl transfer, completely eliminated the translocation of the wild-type ribosome, whereas the A1067U mutant still retained half of the activity (Spahn et al., 1996). Both of these regions are highly conserved phylogenetically; the former is located at the bottom

FIGURE 3. Sensitivity of peptidyltransferase activity of 23S rRNA toward antibiotics, RNase digestion, and mutation. A: AcPhe-Phe synthesis was performed in the same manner as described in the legend to Figure 1A in the presence and absence of chloramphenicol and carbomycin. Before the reaction, 23S rRNA was either not treated (lane 1), or treated with 2 mM chloramphenicol (lane 2) or carbomycin (lane 3) at 0°C for 15 min. The concentration of these antibiotics in the stock solution was 500 mM in pyridine and the final concentration of pyridine in both lanes was 0.4%. B: 23S rRNA was either not treated (lane 1), or treated with 0.5 mg/mL RNase A (lane 2) or T1 (lane 3) at 37°C for 30 min. Both RNAs were purified by the conventional procedure with phenol extraction followed by 7 M urea–4% polyacrylamide gel electrophoresis. Using these RNAs, AcPhe-Phe synthesis was performed as described in the legend of Figure 1A. C: AcPhe-Phe formation performed as described in the legend of Figure 1A with the wild-type (G2252) or mutant (G2252U, A1067U, or A1067U/C2507U/G2581A) 23S rRNAs. G2252U transcripts and the template plasmid for T7 polymerase were a kind gift of Dr. R. Green, and the plasmids for A1067U and A1067U/C2507U/G2581A were a kind gift of Dr. C.M.T. Spahn. Lane 3 indicates the control without 23S rRNA. D: Summary of relative activities in A–C for the case where the control activity is 100%. In all of the assays, the pH was always carefully confirmed, even in the controls.
of the peptidytransferase center ring and the latter in domain II. Taking note of their results, we further investigated two mutated 23S rRNA transcripts, A1067U and A1067U/C2507U/G2581A, with respect to their AcPhe-Phe formation in our system. As shown in Figure 3C, we were able to demonstrate that AcPhe-Phe formation was not influenced by the A1067U mutation (yield: 1.9–2.0%), whereas the relative activity with A1067U/C2507U/G2581A decreased to ca. 50% (yield: 0.8–0.9%).

From the results of the experiments using antibiotics, RNase digestions, and mutants of the 23S rRNA, which are summarized in Figure 3D, it can be deduced that peptide bond formation is actually due to the peptidytransferase contained in the 23S rRNA transcript.

Effects of other rRNAs

It is well known that peptidytransferase is located in the ribosomal large subunit, and that codon recognition is performed by the small subunit. We also confirmed that neither 5S nor 16S rRNA has peptidytransferase activity. E. coli 5S and 16S rRNA transcripts were prepared in the same manner as was used for preparing the 23S rRNA transcript, and peptide bond formation reactions were tested based on a similar weight concentration for the three kinds of rRNA. In order to avoid a “molecular crowding” effect arising from a high rRNA concentration, which would increase the effective local concentrations of the reactants and thereby enhance the reaction rates, AcPhe-Phe formation was performed with a lower concentration of rRNAs, i.e., 2.4 mg/mL (final concentration). Because of this lower concentration, the imaging plate needed to be exposed for a week (see Materials and Methods) to obtain distinct results (Fig. 4). In this case, with the background level lowered to 0.05–0.07%, almost the same yields of AcPhe-Phe (0.2–0.3%) were observed in the presence of 23S rRNA (lanes 4–6 in Fig. 4), whereas the other rRNAs without 23S rRNA had no effect. Figure 4 shows only the weight concentration-based result and supports the postulation that peptidytransferase is located in 23S rRNA.

**DISCUSSION**

**Differences between our system and the conventional puromycin reaction**

We have presented experimental results suggesting that unmodified 23S rRNA transcribed in vitro has intrinsic peptidytransferase activity in the complete absence of any ribosomal proteins. In contrast, some recent reports have claimed that modified nucleotides of 23S rRNA (Green & Noller, 1996) and ribosomal proteins (Noller, 1993) are essential for reconstituting activity in the puromycin reaction with a 3’-fragment of AcPhe-tRNA. We consider that our results could arise from a combination of self-folded 23S rRNA, a buffer containing a high concentration of magnesium at a relatively high pH, 0.5% SDS, and intact tRNA rather than a tRNA fragment and puromycin.

In our assay system with naked 23S rRNA, puromycin had no activity. Puromycin binds only weakly to 23S rRNA, because it is much smaller in size than tRNA. It is well known that the puromycin reaction requires a relatively high molar concentration of the antibiotic in relation to that of ribosome: for example, 0.5 mM of puromycin for 1 μM of ribosome (Traut & Monro, 1964). On the other hand, ribosome was found to be fully occupied by Phe-tRNA when its concentration was only 2.5 times that of ribosome (Schilling-Bartetzko et al., 1992). Because we could obtain an AcPhe-Phe yield of only around 2% in our system, even using Phe-tRNA, one reason for the lack of a puromycin reaction was probably weak puromycin binding. Another likely reason is that puromycin binding probably requires some of the ribosomal proteins; this probability is supported by the residual activity of peptide bond formation in our system, even in the presence of chloramphenicol or
carbomycin. These antibiotics might also need some of the ribosomal proteins for effective inhibition.

Further identification of products

Although we tried to further identify the products by chymotryptic digestion and mass spectroscopy, no unambiguous results could be obtained, presumably because the amount of labeled AcPhe-Phe that could be recovered from the TLC plate was insufficient due to strong product adsorption. When peptide bond formation was performed using radiolabeled AcPhe-tRNA and unlabeled Phe-tRNA, we found only the spots corresponding to AcPhe-Phe, AcPhe, and the upper byproduct in Figure 2A. In contrast, the spots for AcPhe-Phe, Phe, and the lower byproduct in Figure 2A were observed in the cases of unlabeled AcPhe-tRNA and labeled Phe-tRNA (data not shown). These results suggest that the upper byproduct was derived from AcPhe-tRNA, and the lower one from Phe-tRNA.

Likely reasons for low yield of AcPhe-Phe

When the yield of AcPhe-Phe is 1%, 1 mol 23S rRNA is calculated to produce only 0.0015 mol peptide bonds. Two reasons can be postulated to explain this very low efficiency. The first is the lower molar amount of active 23S rRNA in the total 23S rRNA transcript due to the absence of some kinds of modification and ribosomal proteins that might help rRNA folding. With regard to tRNA, it has been reported that modified nucleotides make the conformation of tRNA more rigid (for example, Yokoyama et al., 1987; Przykorska, 1995). The conformations of some rRNAs have been investigated, and native and denatured forms of 5S rRNA (Richards et al., 1973) and 16S rRNA (Ungewickell et al., 1977) isolated from E. coli ribosome were shown to be well resolved by gel electrophoresis with magnesium. It was also shown that the denatured conformation of 16S rRNA can be refolded into the native conformation by heating ribosome-derived 16S rRNA at 40 or 60 °C and then slowly cooling it to 0 °C (Ungewickell et al., 1977).

Although no conformer of 23S rRNA was distinguished by electrophoresis, probably due to its long chain length, with the aim of enhancing the folding of the transcript and improving the yield of AcPhe-Phe, we preincubated the 23S transcript in the self-folding buffer at 40 or 60 °C and then either slowly cooled it to 0 °C or kept it at 40 or 60 °C until just before the peptide bond formation assay. Unfortunately, no improvement was observed as a result of the slow cooling, which might be indicative of the importance of the modification. To obtain further conformational information on the 23S rRNA transcript, CD spectra of folded (dialyzed against the self-folding buffer) and denatured (heated at 95 °C and quenched) 23S rRNA transcripts, together with those of the 50S subunit that was used as a completely folded 23S rRNA for reference, were obtained in the 220–320 nm region. The maximal wavelength of a positive peak in the CD spectrum of 23S rRNA shifted from 266 to 267 nm on transition from the folded to denatured state with no detectable change in the band strength, whereas that of the 50S subunit remained at 265 nm (data not shown). This observation implies that the self-folded 23S transcript takes an intermediate state between the denatured and completely folded conformations (Watanabe et al., 1973).

With regard to nucleotide modification, in a two-step reconstitution following the protocol established earlier by Nierhaus and Dohme (1974), in which 23S rRNA, 5S rRNA, and total large-subunit proteins were incubated for 20 min at 44 °C with 4 mM Mg2+ and then for 90 min at 50 °C with a higher (20 mM) magnesium concentration, Green and Noller (1996) recently showed that in vitro-transcribed 23S rRNA formed only inactive 50S-like particles in the presence of the total 50S proteins, and that as many as six base and/or ribose modifications may be required for the assembly of functional 50S subunits. This finding implicating the need for several modified bases in either the assembly or function of the 50S subunit is in agreement with our preliminary experimental results showing that in vivo-transcribed 23S rRNA extracted from 50S subunits had around 2.5-fold the activity of in vitro-transcribed 23S rRNA. The effects of nucleotide modification on rRNA activity will be investigated in a future study.

The second reason that can be postulated to account for the very low AcPhe-Phe yield is deacylation of AcPhe- and Phe-tRNA during the lengthy incubation at a high pH. We found that the half-lives of AcPhe- and Phe-tRNA were 45 and 17 min, respectively, under the conditions we used for peptide bond formation (pH 8.2, 37 °C). Comparison with the time courses of peptide bond formation in Figure 1C shows that Phe-tRNA was too unstable to form AcPhe-Phe adequately. In the cases of labeled AcPhe-tRNA and nonradioactive Phe-tRNA, we also observed that doubling the concentration of Phe-tRNA increased the radioactivity of AcPhe-Phe 1.7–1.8-fold. These findings thus suggest that the low yield could be a consequence of the short lifetime of Phe-tRNA in comparison with the velocity of peptide bond formation.

Mutation studies

Using a tRNA fragment, Noller and his coworkers reported that the U2252 mutation of 23S rRNA eliminated the ribosomal activity of the puromycin reaction almost completely in the presence of ribosomal proteins (Samaha et al., 1995). In our experiment with naked 23S rRNA and native tRNAs, mutant 23S rRNAs possessing mutations at both of the two conserved helices around the peptidytransferase center ring appeared to work at the same relative activity, ca. 50%.
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One of these helices is the eighth helical segment in domain V, formed by the nucleotides from G2246 to C2258; the other is the 16th segment, from U2506 to G2583. We can make two interpretations of this finding. First, it may be that one of the reasons for the remaining activity is that we used native tRNA instead of a tRNA fragment. According to footprinting data, intact tRNA has multiple contact sites with rRNA (Huttenhofer & Noller, 1992), so local mutation of 23S rRNA does not seem to be critical with regard to tRNA–rRNA interaction. Second, from our results, we can only state with surety that these two helices are equally involved in peptide bond formation by 23S rRNA. It may be, however, that the two helices are close to each other in the three-dimensional structure of 23S rRNA. To obtain more convincing results, we are now studying the modified CCA-terminal of tRNA into which mutations that can compensate for the 23S rRNA mutations have been introduced. In a forthcoming report, we also expect to be able to put forward more quantitative arguments on the mutations of both of 23S rRNA and tRNA on the basis of kinetic studies.

Possible structural studies

Recently, three-dimensional structural features of the total ribosome of E. coli (Frank et al., 1995) and its complex with tRNAs (Agrawal et al., 1996) were revealed by cryoelectron microscopy coupled with three-dimensional reconstruction at 25 Å resolution. These three-dimensional visualizations support an earlier model of the E. coli ribosome large subunit in which the peptidyltransferase center is located between its ridge and the central protuberance (Lake, 1982). However, the ribosomal large subunit has seemed to be too large and complex to permit the construction of a three-dimensional structural model of the peptidyltransferase center. In the work reported here, we succeeded in developing an in vitro assay system for monitoring the peptidyltransferase activity of 23S rRNA. This system may enable the minimum region for peptidyltransferase to be identified by fragmentation, stepwise-deletion, and site-directed mutation of 23S rRNA, and, as a result, in the near future it may become possible to build a three-dimensional structural model by using suitable structural analysis methods such as X-ray crystallography and NMR spectroscopy, as is the case for some ribozymes (for example, see Pley, 1994).

MATERIALS AND METHODS

Plasmid constructs

Plasmids containing the 5S, 16S, and 23S rRNA genes from E. coli strain A19, named pEC5SM, pEC16SM, and pEC23SN, respectively, carried the class III T7 promoter fused with the 5′ end of each RNA gene. The BamH I recognition site was located just upstream of the T7 promoter and Pst I was downstream of the 3′ end of each RNA gene, allowing run-off transcription. To obtain high efficiency in the transcription reaction, the class III promoter sequence for T7 RNA polymerase should be followed by a mono-triad of G; the first bases of the 5′ ends of the 5S and 16S rRNA genes were thus respectively replaced by G. Because the 3′ end of the mature rRNA gene was followed immediately by the Pst I site, each run-off transcript possessed an extra C base at its 3′ end.

Preparation of T7 RNA polymerase

The T7 RNA polymerase gene was cloned and expressed using an E. coli BL21/pAR1219 system according to the literature (Davanloo et al., 1984). A large amount of active enzyme was prepared by single-column purification with S-Sepharose Fast Flow (Pharmacia) (Zawadzki & Gross, 1991). About 130 A\textsubscript{260} units (180 mg) of the polymerase were usually obtained from 4 L culture.

Synthesis of E. coli 23S rRNA by run-off transcription in vitro

A 23S rRNA transcript was obtained from linearized pEC23SN by Pst I as a template DNA. First, 100 μL of 10 mg/mL T7 RNA polymerase was added to 9.9 mL reaction mixture containing 40 mM HEPES-KOH, pH 7.8, 5 mM DTT, 2 mM (each) NTP mixture (pH 7.0 with KOH), 20 mM MgCl\textsubscript{2}, 2 mM spermidine, 25 μg/mL bovine serum albumin, 1 U/μL ribonuclease inhibitor from human placenta (Takara), and 0.2 A\textsubscript{260}/mL template DNA, and the reaction mixture was incubated for 45 min at 42°C. The reaction was chased by adding 20 μL of T7 RNA polymerase and incubation for 45 min at 42°C two more times. The transcription was stopped by 1 mL of 500 mM EDTA, pH 8, and the white precipitate of magnesium pyrophosphate was dissolved completely. The transcript was collected by the conventional phenol treatment and EtOH precipitation.

Purification and self-folding of 23S rRNA transcript

We have previously found that Qiagen, an anion-exchange column, offers high performance for the purification of a large amount of product after in vitro transcription. By using a Qiagen column, the 23S rRNA transcript could be isolated from the template DNA and unreacted NTPs in the following manner. Powder dried from the transcription was dissolved in 20 mL QA buffer: 50 mM MOPS-KOH, pH 7.0, 400 mM NaCl, and 15% EtOH. A sample of the solution was applied onto a Qiagen-tip 500 previously equilibrated with QAT buffer: 50 mM MOPS-KOH, pH 7.0, 400 mM NaCl, 15% EtOH, and Triton X-100. The column was washed with 20 mL QA buffer five times, and the transcript was eluted from the column 2 times with QRU buffer: 50 mM MOPS-KOH, pH 7.0, 1 M NaCl, 15% EtOH, and 6 M urea. To be sure that peptide bond formation activity does occur without the help of any ribosomal proteins, the 23S rRNA transcript should be free from any contaminant, such as urea. We therefore dialyzed the recovered transcript against a self-folding buffer, which contained 50 mM HEPES-KOH, pH 7.5,
20 mM Mg(ACO)2 and 400 mM NH4Cl, using a Slide-A-Lyzer 10,000 MW (Pierce). During dialysis, purification and self-folding of the 23S rRNA could be well accomplished at the same time. Because peptide bond formation is sometimes inhibited by the presence of Na+, we used 600 mM K+ as the carrier cation of the alcohol precipitate. When the scale of in vitro transcription was 10 mL, 60 A260 units (2 nmol) of purified 23S rRNA transcript were prepared normally.

Preparation of AcPhe-tRNA^Phe and Phe-tRNA^Phe

tRNA^Phe from E. coli MRE600 (BioGenes) was phenylalanyl-lated with the S100 fraction from E. coli followed by acetylation with acetic anhydride according to the conventional method (Rheinberger et al., 1988). Uncharged tRNA was removed using HPLC with a C4 column (YMC) by elution with 20 mM NH4OAc, pH 5.0, 10 mM Mg(OAc)2, 400 mM NaCl, and 60% MeOH. In order to avoid transesterification of the phenylalanyl moiety in AcPhe- and Phe-tRNAs^Phe with ethanol, the concentration of AcPhe- and Phe-tRNAs^Phe and buffer exchange were performed by centrifugation (Microcon- and Cetricon-10, Amicon) instead of the usual ethanol precipitation. The final stock solution contained 15,000 cpm/μL Ac^14C]Phe- or [14C]Phe-tRNAs in 20 mM KOAc, pH 5.5, and 5 mM MgCl2.

Peptide bond formation

AcPhe-Phe synthesis was performed at 37°C for 2 h in 25 μL of a reaction mixture containing 1.1 pmol/μL Ac^14C]Phe-tRNA^Phe and [14C]Phe-tRNA^Phe from E. coli (specific activity: 9.2 × 10^-4 pmol/cpm), 1.0–7.2 pmol/μL 23S rRNA, 50 mM HEPES-KOH, pH 8.2, 100 mM MgCl2, and 0.5% SDS. After incubation, the reaction was stopped and the aminoacyl bond was hydrolyzed by heating for 15 min at 95°C. The products contained in 20 μL of the reaction mixture supernatant were analyzed by TLC [silica gel plate, (Merck), developed with nBuOH/ACOH/H2O = 4/0.9/1], followed by radioactive detection on an imaging plate (Fuji Photo Film). Continuous exposure for up to two days was needed under high shielding with lead plates. Radioactivity of 500 cpm was also spotted at the corner of the TLC plate and exposed at the same time. We confirmed the photographic reproduction and converted the primary values of the imaging plate to radioactivity using this control radioactivity. The control radioactive activity was adjusted to the same primary level in each of the graphic figures. The yield of [14C]AcPhe-Phe was calculated as (radioactivity of the spot corresponding to AcPhe-Phe)/ (total radioactivity of all the spots in a lane). Because a slight amount of precipitate was found when 23S rRNA at concentrations higher than 5.0 pmol/μL was incubated at or below room temperature, all the procedures were performed above 30°C to avoid the precipitation of 23S rRNA and/or the products of peptide bond formation with SDS. UV measurement at 260 nm of the supernatant obtained after spinning the reaction mixture showed that, when the incubation temperature was above 30°C, more than 90% of 23S rRNA remained in solution even at 23S rRNA concentrations higher than 5.0 pmol/μL. Also, no changes were observed in absorption spectra obtained at 350–600 nm before and after centrifugation, suggesting no light scattering by any precipitate. In addition, 4% acrylamide gel electrophoresis with 100 mM MgCl2 and 0.5% SDS gave a single band in the centers of the gels with no material remaining in the wells, confirming that the 23S rRNA remained in solution.

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