Ribosomal P0, P1, and P2 proteins, together with the conserved domain of 28 S rRNA, constitute a major part of the GTPase-associated center in eukaryotic ribosomes. We investigated the mode of assembly in vitro by using various truncation mutants of silkworm P0. When compared with wild type (WT)-P0, the C-terminal truncation mutants CAΔ65 and CA81 showed markedly reduced binding ability to P1 and P2, which was offset by the addition of an rRNA fragment covering the P0-P1-P2 binding site. The mutant CAΔ107 lost the P1/P2 binding activity, whereas it retained the rRNA binding. In contrast, the N-terminal truncation mutants NAΔ21–NAΔ92 completely lost the rRNA binding, although they retained P1/P2 binding capability, implying an essential role of the N terminus of P0 for rRNA binding. The P0 mutants NAΔ6, NA14, and CA18-CA81, together with P1/P2 and eL12, bound to the Escherichia coli core 50 S subunits deficient in L10/L17/L12 complex and L11. Analysis of incorporation of 32P-labeled P1/P2 into the 50 S subunits dependent on eukaryotic elongation factors. These results suggested that two P1-P2 dimers bind to close but separate regions on the C-terminal half of P0. The results were further confirmed by binding experiments using chimeric P0 mutants in which the C-terminal 81 or 107 amino acids were replaced with the homologous sequences of the archaeobacterial P0.

The ribosomal large subunits from all organisms contain an active site termed the “GTPase-associated center” that is responsible for the GTPase-related events in protein biosynthesis. This active site is composed of the two highly conserved domains around 1070 and 2660 (Escherichia coli numbering is used throughout) of 23 S/28 S rRNA and the ribosomal proteins bound to the 1070 region (1–3). The protein components of this site in prokaryotic ribosomes constitute a characteristic pentameric complex, L10(L7/L12)2(L7/L12)2 (4, 5), in which two L7/L12 homodimers bind to the C-terminal regions of L10 (6) and constitute a highly flexible and functionally important lateral protuberance, the so-called “stalk” (7). Although the ribosomal stalk is observed by cryo-electron microscopy (8), the detailed structure of this pentameric complex has not been resolved by x-ray crystallography of ribosomes (9–11). The chemical features of protein-protein and protein-rRNA interactions in the GTPase-associated center remain to be clarified.

The animal ribosomal phosphoproteins P0 and P1/P2 (P proteins) are counterparts of prokaryotic L10 and L7/L12, respectively, although P1 and P2 are related but different proteins, unlike prokaryotic L7/L12 (12–14). In yeast cells, there are two P1-type proteins, P1α and P1β, and two P2-type proteins, P2α and P2β (15). It is believed that P proteins constitute a pentameric complex, designated here as P0-P1-P2, in the GTPase-associated center of eukaryotic ribosomes (16, 17). This complex binds not only to eukaryotic 28 S rRNA but also cross-binds to prokaryotic 23 S rRNA and determines the specificity of the ribosome for eukaryotic elongation factor 1α (eEF-1α) and 2 (eEF-2) (19). This strong dependence on the P0-P1-P2 complex for the factor accessibility suggests the direct interaction between the protein complex and elongation factors. It has also been suggested that the P0-P1-P2 complex modulates the functional structures of the sarcin/ricin domain around 2660 as well as the 1070 regions of 23 S/28 S rRNA and makes them accessible to eukaryotic elongation factors (20). Knowledge of the molecular details on the assembly of P0, P1, and P2 proteins onto rRNA is essential to clarify protein-dependent function of the GTPase-associated center.

Current biochemical and genetic evidence indicates that P1 and P2 proteins form the heterodimer (16, 21–24) and P1–P2 dimers bind to a specific region within the C-terminal domain of P0 (25–27). On the other hand, the rRNA binding site seems to be located within the N-terminal region comprising about 200 amino acids (25), although direct evidence has not been shown. In the case of mammalian counterparts, isolated P0 is insoluble in aqueous solution, but the P1–P2 binding to P0 makes P0 soluble (23, 28). We recently showed that silkworm P0, however, is soluble and useful for biochemical assays (16). By using the silkworm mutants, we demonstrated that binding of P1 and P2 to P0 induced the binding activity of P0 to rRNA (16). It is therefore conceivable that binding of P1-P2 to P0 at its C-terminal region affects the overall structure of P0.

To clarify the individual binding sites for two P1–P2 dimers and for rRNA on P0 in vitro, we here constructed 11 kinds of truncation mutants of silkworm P0 and used them for protein–protein and protein–rRNA binding experiments. We identified two neighboring sites for rRNA binding at the N terminus of P0 and suggested that...
the protein-protein and protein-RNA bindings mutually affect each other. To evaluate the in vitro binding data on the basis of ribosome function, we used a hybrid ribosome system developed previously (19) in which E. coli L10-L7/L12 complex and L11 on the 50 S subunit were replaced with the eukaryotic counterparts P0-P1-P2 complex and eL12, respectively. Whenever efficient RNA binding could be observed, complexes containing the truncated P0 mutants bound to E. coli core ribosomes and induced activities dependent on eukaryotic elongation factors. It is interesting that ribosomes carrying a P0 variant accessible to only one P1-P2 heterodimer retained reduced but significant activity.

**MATERIALS AND METHODS**

**Plasmid Construction, Protein Expression, and Purification**—The cDNAs for Bombyx mori ribosomal proteins P0, P1, P2, and eL12 (a eukaryotic homologue of prokaryotic L11) were provided by Dr. K. Mita (National Institute of Agrobiological Sciences). The coding region in each cDNA was amplified by PCR (29), inserted to E. coli expression vector pET28c or pET3a (Novagen), and cloned. Proteins expressed in E. coli cells were purified, as described previously (16). The DNA fragments coding for various truncated P0 were also amplified by PCR using cDNA encoding full-length (WT, 1–316 amino acids) of P0 as a template (see Fig. 1). The overlapping PCR method (30) was used to construct the chimeric P0 mutants composed of the N-terminal 1–235 (CA81) and 1–209 (CA107) amino acid sequences of silkworm P0 fused to the C-terminal sequences of the archaeabacterial (Pyrococcus horikoshii) P0-like protein, which are homologous to the 236–316 and 210–316 sequences of silkworm P0, respectively. The genomic DNA was used as a template of PCR to amplify the DNA fragments for the two C-terminal amino acid sequences of P. horikoshii P0. The DNA fragment was cloned into pET28c, and the P0 mutant was expressed and purified as described above.

*In Vitro RNA Synthesis*—The rat rDNA fragment containing residues 1841–1939 that correspond to 1029–1127 of 18S (designated here the 1070 domain) was amplified by PCR and inserted into the HindIII and XbaI sites of pSPT 18 (Roche Applied Science). The RNA fragment was synthesized using the plasmid DNA and SP-6 RNA polymerase and purified, as described previously (31).

**P0-P1-P2 Complex Formation**—After the amounts of isolated proteins were determined with the Micro BCA protein assay reagent kit (Pierce), the concentrations of individual protein samples (pmol/μl) were estimated considering that 1 μg of WT-P0 (or P0 mutants), P1, and P2 correspond to 29.3 (or 26.6–42.2), 87.3, and 86.6 pmol, respectively. The protein samples were mixed together at a molar ratio of P0 sample: P1: P2 of 1:3:3, and the complex was reconstituted, as described previously (16). The P0-P1-P2 complex formation in the presence or absence of a 2-fold excess of the rRNA fragments of the 1070 domain was confirmed by gel electrophoresis at 6.5 V/cm with a buffer system containing 5 mM MgCl2, 50 mM KCl, and 50 mM Tris-HCl (pH 8.0). Samples were electrophoresed for 6 h at constant voltage and 4 °C with buffer recirculation (16). The gel was stained with Coomassie Brilliant Blue.

**Ribosomal Subunits and the 50 S Core Particles**—E. coli ribosomal subunits were prepared as described previously (20). 50 S cores deficient in L10-L7/L12 and L11 were prepared by extraction of the 50 S subunits from the L11-deficient E. coli mutant AM68 (32) in a solution containing 50% ethanol and 0.5 M NH4Cl solution at 0 °C, as described previously (19).

**Gel Retardation**—[32P]RNA fragments covering the 1070 domain (5 pmol) synthesized as described above were mixed with 10 pmol of P0-P1-P2 complex sample and 10 pmol of eL12 (28) and incubated at 30 °C for 5 min in 10 μl of a solution containing 20 mM MgCl2, 300 mM KCl, 20 mM Tris-HCl, pH 7.6 (16). The RNA-protein complexes were separated by 6% polyacrylamide native gel, as described above. The gel was dried and subjected to autoradiography.

Acrylamide/Agarose Composite Gel Electrophoresis—50 S core particles (10 pmol) were mixed with various P0-P1-P2 complex samples (20 pmol each) together with eL12 (20 pmol). The samples were analyzed by electrophoresis on acrylamide/agarose composite gel composed of 3% acrylamide gel/agarose (acrylamide/bisacrylamide ratio 19:1) and 3% agarose (33), as described previously (19). The gel was stained with Azur B. Binding of proteins to the 50 S core particles was revealed by gel mobility shift.

**Quantitative Analysis of P1 and P2 Incorporated into the Ribosome**—Isolated P1 and P2 (25 μg of each) were incubated with 500 units of casein kinase II (New England Biolabs) and 5 nmol of (32P)ATP (250 μCi/μmol) for 30 min at 30 °C in a solution (50 μl) containing 50 mM KCl, 10 mM MgCl2, 20 mM Tris-HCl, pH 7.5. The 32P-labeled P1 (86 cpm/pmol) was mixed with non-labeled P0 (or CA81) and P2, and the complex was reconstituted as described above. For the E. coli 50 S core (78 pmol), excess amounts (195 pmol) of the P0 32P-P1-P2 complex were added, together with 154 pmol of eL12. The sample was then layered on a 10–28% sucrose gradient in a solution containing 50 mM NH4Cl, 5 mM MgCl2, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.6, and fractionated after centrifugation at 40,000 rpm and 4 °C for 3 h in a Hitachi P-45 ST rotor. The 50 S fraction was collected, and the amount of the associated P1 was estimated by its radioactivity. The 32P-labeled P1 (205 cpm/pmol) was mixed with non-labeled P0 (or CA81) and P1, and its incorporation into 50 S core particles was analyzed as described for 32P-labeled P1.

**Ribosome Functional Assays**—Eukaryotic elongation factors eEF-1α and eEF-2 were isolated from pig liver as described by Iwasaki and Kaziro (34). eEF-2-dependent GTPase and polyphenylalanine synthesis by using poly(U), eEF-1α, and eEF-2 were assayed according to our previous report (16).

**RESULTS**

**Preparation of Various Truncated Mutants of Animal Ribosomal Protein P0**—The eukaryotic ribosomal protein P0 plays a central role in the assembly of the active GTPase-associated center. Here we investigated the structural elements required for binding of two P1/P2 dimers and rRNA by using truncated mutants that are summarized in Fig. 1A. All P0 mutants including five C-terminal mutants (CA18, CA55, CA65, CA81, and CA107) and six N-terminal truncation mutants (NA6, NA14, NA21, NA48, NA66, and NA92) were expressed in E. coli cells but found to be insoluble. The proteins were solubilized in 6 M urea and purified by using ion exchange high pressure liquid chromatography. The purity of all the isolated P0 samples, P1, P2, and eL12, is shown in Fig. 1B. By adding P1 and P2 to individual P0 samples, P0-P1-P2 complexes were reconstituted as described under “Materials and Methods” and used in the following experiments.

**Effect of the C-terminal Truncation of P0 on P0-P1-P2 Assembly**—The formation of P0-P1-P2 complexes was examined by native polyacrylamide gel electrophoresis (Fig. 2A). The complex of WT-P0 with P1 and P2 was detected as a shifted band (Fig. 2A, lane 1), in a similar manner as the authentic proteins from silkworm ribosomes (16). The complexes were also formed efficiently even with P0 mutants, the C-terminal amino acids of which, 299–316 (CA18, lane 2) and 262–316 (CA55, lane 3), were truncated. However, only weak smearing bands were observed in the complexes with the CA65 (lane 4) and CA81 (lane 5) mutants of P0. No complex formation was detected in the sample with the CA107
To eliminate a possibility that the effects of the truncation shown in Fig. 2 result from severe alteration of the tertiary structure of P0 rather than from deletion of the binding sites for P1/P2, we also performed binding experiments using chimeric P0 instead of truncation mutants. Because archaeabacterial (P. horikoshii) ribosomes contain a eukaryotic mutant (lane 6). Each protein mixture was tested for rRNA binding by gel mobility shift assay using a small amount of the $^{32}$P-labeled RNA fragment covering the 1070 region (Fig. 2B). Strong RNA binding was observed for all reconstituted complexes (Fig. 2B, lanes 2–6). The CΔ107 mutant, which had no binding ability to P1/P2, showed reduced but significant binding affinity to the RNA (lane 7). The same mobility shift was observed in the isolated CΔ107 without P1 and P2 (not shown), suggesting that the CΔ107 mutant retains rRNA binding. This is an unexpected result because the WT-P0 fails to bind rRNA without P1 and P2 (16).

The same experiment as Fig. 2A was performed after the addition of an excess amount of the RNA fragment (Fig. 2C). In the presence of the rRNA fragment, bands of the P0-P1-P2 complexes with all the P0 samples except CΔ107 were more distinct (Fig. 2C). Particularly, the complexes with CΔ65 and CΔ81 were stabilized markedly with the RNA fragment (Fig. 2C, lanes 4 and 5). The complex formation of CΔ107 with P1-P2 was not detected even after the addition of the RNA (lane 6). To confirm that the complexes formed in Fig. 2C all contain P0 (or its mutants), P1 and P2, the bands of the complexes were cut out of the gel and subjected to SDS gel electrophoresis. The gel was stained with a fluorescent dye, SYPRO Orange, to quantitate roughly the relative amounts of P1/P2 by fluorescence intensity (Fig. 2D). The three protein components were detected in all complexes formed. The intensity of both P1 and P2 in the complexes with CΔ65 and CΔ81 (lanes 4 and 5) was approximately half of that in the other complexes (lanes 1–3).

To eliminate a possibility that the effects of the truncation shown in Fig. 2 result from severe alteration of the tertiary structure of P0 rather than from deletion of the binding sites for P1-P2, we also performed binding experiments using chimeric P0 instead of truncation mutants. Because archaeabacterial (P. horikoshii) ribosomes contain a eukaryotic protein, could form a complex with silkworm P1/P2 (lane 15). The same experiment as Fig. 2C was performed after the addition of an excess amount of the RNA fragment covering the 1070 domain (lane 1, RNA alone), separated by native gel electrophoresis, and then subjected to autoradiography. The complexes formed in the absence of the rRNA fragment (lanes 1–3) and in its presence (lanes 4–6) were analyzed by native gel electrophoresis, as shown in Fig. 2A and C.

P0-like protein that does not cross-bind to silkworm P1/P2, we replaced the C-terminal 107-amino acid sequence of which is from the corresponding region of the archaebacterium P. horikoshii. The complexes were reconstituted by mixing P1-P2 with WT-P0 (lanes 1), CΔ18 (lane 2), CΔ55 (lane 3), CΔ65 (lane 4), CΔ81 (lane 5), and CΔ107 (lane 6) as described under “Materials and Methods,” and each sample containing 100 pmol of P0 was subjected to native gel electrophoresis. The gel was stained with Comassie Brilliant Blue. B, the P0-P1-P2 complex samples (1 μg of each) containing WT-P0 (lane 2), CΔ18 (lane 3), CΔ55 (lane 4), CΔ65 (lane 5), CΔ81 (lane 6), and CΔ107 (lane 7) were mixed with 5 pmol of $^{32}$P-labeled RNA fragment covering the 1070 domain (lane 1, RNA alone), separated by native gel electrophoresis, and then subjected to autoradiography. C, the same samples as in A were incubated with 200 pmol of non-labeled RNA fragment covering the 1070 domain and performed for the same gel analysis. The gel was stained with SYPRO Orange D, the bands corresponding to the protein-RNA complexes observed in C were cut out. After treatment in SDS sample solution, gel pieces were loaded onto SDS gel as samples. After SDS gel electrophoresis, gel was stained with SYPRO Orange (Amer sham Biosciences) and observed by STORM 860 PhosphorImager (Amer sham Biosciences).

FIGURE 1. Preparations of silkworm ribosomal proteins and P0 truncation mutants. A, truncation mutants of P0 used in this study. DNA fragments coding individual regions of silkworm P0 were amplified and cloned into an E. coli expression vector. B, SDS gel electrophoretic pattern of isolated protein samples (2 μg of each): lane 1, WT-P0; lane 2, CΔ18; lane 3, CΔ55; lane 4, CΔ65; lane 5, CΔ81; lane 6, NΔ66; lane 7, NΔ66; lane 8, NΔ144; lane 9, NΔ21; lane 10, NΔ48; lane 11, NΔ66; lane 12, NΔ92; lane 13, P1; lane 14, P2; lane 15, el12.

FIGURE 2. Effect of C-terminal truncation of P0 on the P0-P1-P2 complex formation. A, the complexes were reconstituted by mixing P1-P2 with WT-P0 (lane 1), CΔ18 (lane 2), CΔ55 (lane 3), CΔ65 (lane 4), CΔ81 (lane 5), and CΔ107 (lane 6) as described under “Materials and Methods,” and each sample containing 100 pmol of P0 was subjected to native gel electrophoresis. The gel was stained with Coomassie Brilliant Blue. B, the P0-P1-P2 complex samples (1 μg of each) containing WT-P0 (lane 2), CΔ18 (lane 3), CΔ55 (lane 4), CΔ65 (lane 5), CΔ81 (lane 6), and CΔ107 (lane 7) were mixed with 5 pmol of $^{32}$P-labeled RNA fragment covering the 1070 domain (lane 1, RNA alone), separated by native gel electrophoresis, and then subjected to autoradiography. C, the same samples as in A were incubated with 200 pmol of non-labeled RNA fragment covering the 1070 domain and performed for the same gel analysis. The gel was stained with SYPRO Orange D, the bands corresponding to the protein-RNA complexes observed in C were cut out. After treatment in SDS sample solution, gel pieces were loaded onto SDS gel as samples. After SDS gel electrophoresis, gel was stained with SYPRO Orange (Amer sham Biosciences) and observed by STORM 860 PhosphorImager (Amer sham Biosciences).
P1/P2 are comparable with those of the CΔ81 and CΔ107 P0 mutants (Fig. 2C). It should be added that the chimeric P0, the C-terminal 107-amino acid sequence of which is from P. horikoshii, could bind the P. horikoshii stalk dimers and form a functional complex.1 These results supported the binding data with the C-terminal truncation mutants.

Effect of the N-terminal Deletion of P0 on P0-P1-P2 Assembly—The formation of P0-P1-P2 complexes was also examined with the N-terminal truncation mutants of P0 (Fig. 4A). Unlike the C-terminal truncation, all the N-terminal mutants NΔ6–NΔ92 formed complexes that appeared as distinct bands in the presence of P1-P2 (lanes 2–7). The SDS gel electrophoretic analysis of the complexes showed that all contained P0 (or its mutants), P1, and P2 (data not shown), suggesting that the N-terminal truncations did not disrupt the binding potentials between P0 and P1-P2. In contrast, the N-terminal deletions caused marked effect of the rRNA binding (Fig. 4B). When N-terminal amino acids 1–6 (NΔ6) were truncated, the rRNA binding ability of P0-P1-P2 complex was reduced (Fig. 4B, lane 3). By further deletions (NΔ14–NΔ92, lanes 4–8), the binding ability was lost. However, the rRNA bindings of the complexes with NΔ6 and NΔ14, but not with NΔ21–NΔ92, were recovered by the addition of eL12 (data not shown).

Assembly of the Truncated P0 Mutants onto the E. coli 50 S Particles—Unlike prokaryotic L10, eukaryotic P0 was hardly released from the animal 60 S subunit by standard high salt/ethanol conditions, and reconstitution experiments of the GTPase-associated center with animal ribosomes are much harder than those with E. coli ribosomes. We have established conditions to form a hybrid ribosomal particle in which L10/L7/L12 complex and L11 within the E. coli 50 S subunit are replaced with animal P0-P1-P2 and eL12, respectively (19). We therefore used the E. coli 50 S subunits to investigate the assembly of animal P0-P1-P2 and

![Image](image-url)

FIGURE 4. Effect of N-terminal truncation of P0 on the P0-P1-P2 complex formation. A, the complexes were reconstituted by mixing of P1/P2 with WT-P0 (lanes 1), NΔ6 (lanes 2), NΔ14 (lane 2), NΔ21 (lane 4), NΔ48 (lane 5), NΔ66 (lane 6), and NΔ92 (lane 7), and each sample (10 μg) was subjected to native gel electrophoresis. The gel was stained with Coomassie Brilliant Blue. B, the POP1-P2 complex samples (1 μg each) containing WT-P0 (lane 2), NΔ6 (lane 3), NΔ14 (lane 4), NΔ21 (lane 5), NΔ48 (lane 6), NΔ66 (lane 7), and NΔ92 (lane 8) were mixed with 5 pmol of [32P]RNA fragment covering the 1070 domain (lane 1, RNA alone), separated by native gel electrophoresis, and then subjected to autoradiography.

![Image](image-url)

FIGURE 5. Effect of the P0 truncation on incorporation of the P. P1-P2 complexes to E. coli 50 S core particles. A, the 50 S core particles deficient in L10/L7/L12 and L11 (10 pmol, lane 1) were incubated with eL12 (20 pmol, lane 2) and the same amount of eL12, together with the P0-P1-P2 complexes (20 pmol each), containing WT-P0 (lane 3), CΔ18 (lane 4), CΔ55 (lane 5), CΔ65 (lane 6), CΔ81 (lane 7), and CΔ107 (lane 8) and analyzed by electrophoresis on acrylamide-agarose composite gel electrophoresis, as described under “Materials and Methods.” B, the 50 S core particles (10 pmol, lane 1) were incubated with eL12 (20 pmol, lane 2) and the same amount of eL12, together with the P0-P1-P2 complexes (20 pmol each), containing WT-P0 (lane 3), NΔ6 (lane 4), NΔ14 (lane 5), NΔ21 (lane 6), NΔ48 (lane 7), NΔ66 (lane 8), and NΔ92 (lane 9) and analyzed as A.

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FIGURE 6. Quantitative analysis of P1 (A) and P2 (B) bound to WT-P0 (A and B, left) and the CΔ81 mutant (A and B, right) by sucrose density gradient centrifugation. A, the 50 S core particles (78 pmol) were mixed with excess amounts of PO-P1-P2 complexes consisting of [32P]P1 (86 cpm/pmol P1), non-labeled P2, and WT-P0 (left) or the CΔ81 mutant (right), together with eL12. The samples were separated by sucrose gradient centrifugation, as described under “Materials and Methods.” The amount of P1 incorporated to the 50 S core was estimated by radioactivity of P1. B, the same experiment as in A was performed by using PO-P1-P2 complexes consisting of [32P]P2 (105 cpm/pmol P2), non-labeled P1, and WT-P0 (left) or the CΔ81 mutant (right).
Molecular Dissection of Animal Ribosomal Protein Po

Two P1-P2 Heterodimers Bind to the Restricted C-terminal Regions of Po—It has been shown that the C-terminal fragment of E. coli L10, composed of amino acid residues 71–164, participates in bindings of two L7/L12 dimers (35). More recently, Griaiznova and Traut (6) have clarified that 20 amino acids at the C-terminus of E. coli L10 determine the binding sites for all four copies of L7/L12 proteins, i.e., the region of amino acids 145–154 is involved in the binding of one L7/L12 dimer, and the following amino acid sequence 155–164 is for another L7/L12 dimer (Fig. 8A). After completion of this manuscript, Diaconu et al. (37) reported the crystal structure of the L10/L7/L12 complex in Thermotoga maritima, and the results surprisingly revealed three repetitive binding sites for L7/L12 dimers on the discrete C-terminal helix α-8. In yeast, the regions including residues 230–290 (25), 212–262 (26), and 213–250 (27) within the C-terminal half of Po have been identified as sites for P1/P2 binding (Fig. 8A), although individual sites for two P1/P2 dimers could not be resolved.

Our present evidence suggested that there are two neighboring and distinct binding sites for P1-P2 in the C-terminal half of silkworm Po, i.e., the regions of residues 252–261 and 210–251 of silkworm P0 bind either one P1-P2 heterodimer. To compare the present data with those in E. coli (6), amino acid sequences of the C-terminal halves of silkworm (B. mori) Po and E. coli L10 are aligned, together with yeast (S. cerevisiae) and human (Hs) P0 (Fig. 8A), basically according to Shimmin et al. (37), who compared the sequences of L10 equivalent proteins from eubacteria, archaeabacteria, and eukaryotes. In this alignment, the region of residues 252–261 of silkworm P0, participating in binding of P1–P2 at the C-terminal side, neatly corresponded to the sequence 155–164 of E. coli L10, required for binding of one L7/L12 homodimer at the C-terminal side.

DISCUSSION

Two P1-P2 Heterodimers Bind to the Restricted C-terminal Regions of Po—It has been shown that the C-terminal fragment of E. coli L10, composed of amino acid residues 71–164, participates in bindings of two L7/L12 dimers (35). More recently, Griaiznova and Traut (6) have clarified that 20 amino acids at the C-terminus of E. coli L10 determine the binding sites for all four copies of L7/L12 proteins, i.e., the region of amino acids 145–154 is involved in the binding of one L7/L12 dimer, and the following amino acid sequence 155–164 is for another L7/L12 dimer (Fig. 8A). After completion of this manuscript, Diaconu et al. (37) reported the crystal structure of the L10/L7/L12 complex in Thermotoga maritima, and the results surprisingly revealed three repetitive binding sites for L7/L12 dimers on the discrete C-terminal helix α-8. In yeast, the regions including residues 230–290 (25), 212–262 (26), and 213–250 (27) within the C-terminal half of Po have been identified as sites for P1/P2 binding (Fig. 8A), although individual sites for two P1/P2 dimers could not be resolved.

Our present evidence suggested that there are two neighboring and distinct binding sites for P1-P2 in the C-terminal half of silkworm Po, i.e., the regions of residues 252–261 and 210–251 of silkworm P0 bind either one P1-P2 heterodimer. To compare the present data with those in E. coli (6), amino acid sequences of the C-terminal halves of silkworm (B. mori) Po and E. coli L10 are aligned, together with yeast (S. cerevisiae) and human (Hs) P0 (Fig. 8A), basically according to Shimmin et al. (37), who compared the sequences of L10 equivalent proteins from eubacteria, archaeabacteria, and eukaryotes. In this alignment, the region of residues 252–261 of silkworm P0, participating in binding of P1–P2 at the C-terminal side, neatly corresponded to the sequence 155–164 of E. coli L10, required for binding of one L7/L12 homodimer at the C-terminal side.
The region of residues 210–251 of P0, identified as site-essential for another P1-P2 binding, covered the site corresponding to region of residues 145–154 of L10, which is required for the binding of another L7/L12 dimer. There may be an analogous feature in interaction between L10 equivalent proteins and the stalk dimers, although the amino acid sequence identity of the putative binding site for the stalk dimers in the L10 orthologs are very low.

The N Terminus of P0 Is Crucial for rRNA Binding—It has been reported that E. coli L10 and L10/L12 complex bind directly to the 1070 region of 23 S rRNA (3, 38, 39), and the L10 fragment lacking the N-terminal 1–69 amino acids fails to assemble into the ribosome, together with L7/L12 (35). In eukaryotes, the yeast P0 variant lacking the N-terminal 1–69 amino acids fails to assemble into the ribosome, with eL12. The present results strongly suggested that the N terminus of P0 including residues 1–21, particularly residues 15–21, plays an essential role in binding of the protein complex to the 1070 rRNA domain.

A possible alignment of the N-terminal amino acid sequences of P0 homologues among five eukaryotic species is shown in Fig. 8B. There are highly conserved amino acids Lys-10, Tyr-13, Phe-14, and Lys-16 (BmP0 numbering). Two conserved acidic amino acids Asp/Glu-22 and Asp/Glu-23 are also notable. These amino acids seem to constitute at least a part of the RNA binding site in P0. In the currently published crystal data of an L10-like protein (36), this N-terminal region (residues 8–21 of BmP0) corresponds to helix a1 that contacts rRNA.

Cooperative Relation among Bindings of two P1-P2 Dimers and rRNA to P0—As discussed above, silkworm P0 protein seems to have two binding sites for P1-P2 heterodimers defined by residues 210–251 and 252–261. The P0 truncation mutants CΔA18 and CΔ55, as well as WT-P0, which appear to retain the two binding sites, form a stable complex with P1 and P2, whereas CΔ65 and CΔ81, which seem to have a single binding site, show only weak binding property to P1-P2 in the absence of the RNA to which P0 binds (Fig. 24). These findings indicate that the residues 210–251-mediated P1-P2 binding is markedly enhanced in the presence of residues 252–261 and binding of the latter to another P1-P2 dimer. There may be two possible explanations for the cooperativity of the binding of the two P1-P2 dimers: 1) the 252–261-mediated binding of P1-P2 may cause a conformational change of P0 that stimulates the 210–251-mediated binding and 2) the pentameric complex may be stabilized by interactions between the two P1-P2 dimers through P1-P1 and P2-P2 interactions, which has been suggested by previous biochemical assays (41, 42) and two hybrid assays (15, 21), although the latter assay shows that efficiency of P1-P1 and P2-P2 interactions is much lower than that of P1-P2 interaction.
The present study also shows that the 210–251-mediated P1-P2 binding of P0 is stabilized by its rRNA binding (Fig. 2C). On the contrary, strong rRNA binding of P0 is induced by P1/P2 binding to P0, as described previously (16). Furthermore, the present study shows that the truncation of two P1-P2 binding sites of P0 (CΔ107) increases its rRNA binding ability without P1/P2 (Fig. 2B) and see "Results". These results indicate a correlation between P1-P2 binding to the C-terminal regions of P0 and rRNA binding to the N-terminal region. The rRNA binding site in P0 may be blocked with a part of the P1-P2 binding sites, when P1 and P2 are absent. It is also likely that the N-terminal end constituting a part of the rRNA binding site affects a state of the P1-P2 binding site. It is generally believed that the ribosomal stalks, eukaryotic P0, and prokaryotic L7/L12 participate in interaction with translation factors. The present study suggested an additional role of P1/P2 as a modulator in P0 binding to rRNA.

The Significance of the Presence of Two P1-P2 Dimers in the Ribosome—It is interesting that the hybrid ribosome carrying C₈A₁, which binds only one P1-P2 dimer, retains reduced but significant activity dependent on eukaryotic elongation factors (Fig. 7). The results indicate that amino acid residues 236–316 of P0 including one of the two P1-P2 binding sites and the C terminus homologous to that of P1 and P2 (13) are not essential for basal ribosome activity and suggest that two P1-P2 dimers are required for full activity of both GTPase and polyphenylalanyl-synthetic. A dispensable feature of one of two stalk dimers has also been shown in E. coli L7/L12 (6, 43, 44). We infer that, at least in eukaryotes, the presence of two P1-P2 dimers in the ribosome improves efficiencies of GTPase turnover and thus the elongation cycle, as well as the efficiency of the assembly of the GTPase-associated center, as described above.

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