Deletion of C-Terminal Residues of *Escherichia coli* Ribosomal Protein L10 Causes the Loss of Binding of One L7/L12 Dimer: Ribosomes with One L7/L12 Dimer Are Active†

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ABSTRACT: *Escherichia coli* ribosomal protein L10 binds the two L7/L12 dimers and thereby anchors them to the large ribosomal subunit. C-Terminal deletion variants (Δ10, Δ20, and Δ33 amino acids) of ribosomal protein L10 were constructed in order to define the binding sites for the two L7/L12 dimers and then to make and test ribosomal particles that contain only one of the two dimers. None of the deletions interfered with binding of L10 variants to ribosomal core particles. Deletion of 20 or 33 amino acids led to the inability of the proteins to bind both dimers of protein L7/L12. The L10 variant with deletion of 10 amino acids bound one L7/L12 dimer in solution and when reconstituted into ribosomes promoted the binding of only one L7/L12 dimer to the ribosome. The ribosomes that contained a single L7/L12 dimer were homogeneous by gel electrophoresis where they had a mobility between wild-type 50S subunits and cores completely lacking L7/L12. The single-dimer ribosomal particles supported elongation factor G dependent GTP hydrolysis and protein synthesis in vitro with the same activity as that of two-dimer particles. The results suggest that amino acids 145−154 in protein L10 determine the binding site ("internal-site") for one L7/L12 dimer (the one reported here), and residues 155−164 ("C-terminal-site") are involved in the interaction with the second L7/L12 dimer. Homogeneous ribosomal particles containing a single L7/L12 dimer in each of the distinct sites present an ideal system for studying the location, conformation, dynamics, and function of each of the dimers individually.

A pentameric structure comprised of two protein dimers and a single-copy protein is found in large ribosomal subunits from eubacteria (*1*), eukaryotes (*2*), and archae (*3*). In *Escherichia coli* the pentameric complex named L8 is composed of two dimers of proteins L7/L12 bound to protein L10 (*4*). The complex can be extracted from the ribosome (*5*), and it readily reassociates with ribosomal core particles that lack the complex (*6*). The L8 pentamer can easily be reconstituted from purified components, L10 and L7/L12, and modified variants thereof (*5, 7, 8*). Protein L7/L12 is the only protein that is present in more than a single copy per *E. coli* ribosome (*9, 10*), and it plays a key role in the interaction of soluble translation factors with the ribosome (*11*−*13*). Each L7/L12 dimer is integrated into the ribosome through binding of its α-helical N-terminal domain to protein L10 that binds to 23S ribosomal RNA (*14*−*17*) in the thiostrepton domain considered to be involved in ribosome-factor GTPase activities.

The binding, conformational, and functional properties of the L7/L12 dimers have been intensively investigated, but the molecular mechanism by which they promote protein synthesis is still undefined as is the presence of two dimers. The two dimers have been differentiated by several experimental approaches. On the basis of immune electron microscopy of 50S ribosomal subunits, one dimer has an extended straight conformation that comprises the L7/L12 "stalk", and the other has a bent conformation that lies folded on the body of the ribosome (*18*). Recently, cryo-electron microscopy of *E. coli* 70S ribosomes again showed both extended (stalk-like) and internal densities on the body of the 50S subunit, consistent with the earlier results. The extended form was evident in cryo-electron microscopy of ribosome−tRNA complexes (*19*), while the ribosome−EF-G−GDP−fisidic acid complex revealed direct contact between EF-G[1] and L7/L12 on the body of the 50S subunit that was attributed to contact with the folded L7/L12 dimer (*20*). Incubation of ribosomes with monoclonal antibodies to the N-terminal region of L7/L12 resulted in a loss of only one L7/L12 dimer (*21*), shown by immune electron microscopy to be the stalk (*18*). Since the antibodies inactivated the ribosome these particles gave no functional information. Exposure of L7/L12 to denaturing conditions (6 M urea) gave a protein that rebound to ribosomal core particles lacking L7/L12 in only one dimer per particle after high-speed centrifugation of the particles (*22*). The latter results have proven difficult to reproduce. Fractionation of the (L7/L12−L10)−23S rRNA complex by gel filtration sometimes leads

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[1] Abbreviations: EF-G, elongation factor G; L10Δ10, L10Δ20, and L10Δ33, protein L10 variants with C-terminal deletions of 10, 20, and 33 amino acids, respectively; RP HPLC, reversed-phase high-performance liquid chromatography; L7/L12Cys-63, protein L7/L12 variant with cysteine instead of alanine at position 63; ^{125}I-APDP, ^{125}I-*N*-[4-(*p*-azidosalicylamido)butyl]-3-(2′-pyridyldithio)propionamide.

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to dissociation of one L7/L12 dimer from the complex (*14*). These results indicate nonequivalence of the binding sites for the two dimers and led to the concept of strong and weak binding sites. It was also shown that the dimers do not "jump" from one site to the other. Homogeneous ribosomal particles that contain only one of the two L7/L12 dimers would provide an ideal system for investigating the properties of each of the dimers separately.

The N-terminal fragment of protein L10, residues 1−69, is involved in interaction with the ribosome, and the C-terminal fragment, residues 71−164, binds both L7/L12 dimers (*23*). There are no obvious repeats in the amino acid sequence of L10 (*24*). This implies that the determinants for binding the two L7/L12 dimers are different but gives no clue as to the sequences involved in dimer binding. In the present work, we describe the construction of one of the two possible ribosomal particles with a single L7/L12 dimer using a C-terminal truncated variant of protein L10. The homogeneity of single-dimer ribosomes was established and their functional properties were tested.

MATERIALS AND METHODS

Reagents and Enzymes. Elongation factor G was a gift of Dr. T. Uchiumi (Shinshu University, Japan). [γ-^{32}P]GTP and [^{14}C]-L-phenylalanine were purchased from NEN Life Science Products (Boston, MA), and [^{14}C]iodoacetamide was obtained from Amersham Life Science, Inc. (Arlington, IL). Restriction enzymes were purchased from Promega (Madison, WI), and Vent DNA Polymerase was from New England Biolabs (Beverly, MA).

Plasmids and Bacterial Strains. Plasmid pNF1825 (*25*) was a gift of Dr. P. P. Dennis (The University of British Columbia). Plasmid pET11c (*26*) was purchased from Novagen (Madison, WI). Strain DH5α was used for propagating and maintaining the plasmids. Strain BL21(DE3)-pLysS (*26*) was obtained from Novagen and used for overproduction of recombinant proteins. Ampicillin and chloramphenicol were purchased from Sigma Chemical Co. (St. Louis, MO) and used at concentrations of 100 μg/mL and 34 μg/mL, respectively.

Construction of C-Terminal Deletion Variants of Protein L10. The plasmid pNF1825 was used as a source of *E. coli* L10 operon containing the genes for L10 (*rplJ*) and L7/L12 (*rplL*). Four pairs of oligonucleotide primers were synthesized: (A) GGAGCAAAGCATATGGCTTTAAATC and (B) CCGGAAGGGATCCCTTATTTAAC; (CΔ10) TGCGATTACAGAGTACGAACCAGTTTGC and (DΔ10) CGTACTCTGTAATCGCAGTTATCTTTTTAACG; (CΔ20) TGCGATTATTCTTTCATGGTTGCCATCAGGC and (DΔ20) CATGAAAGAATAATCGCAGTTATCTTTTTAACG; and (CΔ33) TGCGATTAGTAGGTCGGCAGAGTTGCCAGG and (DΔ33) CGACCTACTAATCGCAGTTATCTTTTTAACG. The primer A contained the sequence around the start codon of *rplJ* and had a *Nde*I site introduced upstream of the start codon. Primer B was complementary to the end of *rplL* and it added a *Bam*HI site downstream of the stop codon. Oligonucleotides A and B served as "flanking" primers to coamplify *rplJ* and *rplL*, and *Nde*I and *Bam*HI sites were introduced to clone the genes into expression vector pET11c. First, a recombinant plasmid was constructed to overexpress the gene for wild-type L10. The part of L10 operon containing *rplJ*, the intergenic region, and *rplL* was amplified with primers A and B, digested with *Nde*I and *Bam*HI, and cloned into pET11c.

The C and D oligonucleotides ("deletion" primers) were used to make 3′-end deletions of various length in *rplJ*, and the primers of each pair were designed to have overlapping ends. The deletions were made by the overlap extension approach for PCR mutagenesis (*27*). Three PCRs were the carried out for each construction. First, the truncated *rplJ* genes were amplified by use of a "flanking" primer A and one of the "deletion" primers C. Second, the intact intergene region and full-length *rplL* were amplified together with the "deletion" primer D and a "flanking" primer B. The PCR products AC and DB were annealed, and the joint sequences were amplified using "flanking" primers A and B. The final PCR products (AB) were digested with *Nde*I and *Bam*HI and cloned into pET11c. The deletion mutations and the integrity of the remaining sequences were verified by sequencing of the cloned inserts.

Overexpression and Purification of Wild-Type L10 and Its Truncated Variants. The strain BL21(DE3)/pLysS transformed with one of the recombinant plasmids was grown in 2x LB medium until the A_{600} reached 0.6−0.8; then IPTG was added to a final concentration of 1 mM, and incubation was continued for 3−5 h. Cells were harvested by low-speed centrifugation and disrupted by grinding in alumina (2 g/g of cells). The cell lysate was diluted in a buffer (10 mL/g of cells) containing 10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 7 mM 2-mercaptoethanol and cleared by centrifugation at 30000*g* for 20 min at 4 °C. Wild-type L10, L10Δ10, L10Δ20, and L7/L12 were found soluble in the supernatant fraction (S30), and L10Δ33 remained in the debris fraction. Protein L10Δ33 was solubilized with 70% acetic acid, precipitated with acetone, dissolved in 8 M urea, purified by RP HPLC [Hi-Pore RP 304 column (Bio-Rad, Hercules, CA)] in a gradient of acetonitrile from 0 to 60% in 0.1% TFA and lyophilized. The S30 fraction was centrifuged at 200000*g* for 2 h at 4 °C to pellet the ribosomes. The S200 supernatant fraction was dialyzed overnight against 80 mM NH_4OAc, pH 6.0, with 7 mM 2-mercaptoethanol. Protein L10Δ20 precipitated during dialysis. The precipitate was dissolved in 8 M urea, and L10Δ20 was purified by RP HPLC under the same conditions as those for L10Δ33 and lyophilized. The supernatant fractions after dialysis that contained wild-type L10 and L7/L12, or L10Δ10 and L7/L12 were applied to a Resource Q column (Pharmacia Biotech., Piscataway, NJ) connected to an FPLC system (Pharmacia Biotech.) and equilibrated in the same buffer. The complex of the proteins L10 and L7/L12, or L10Δ10 and L7/L12, was eluted in a gradient of NaCl from 0 to 400 mM in 80 mM NH_4OAc, pH 6.0, with 7 mM 2-mercaptoethanol and then precipitated with acetone. The precipitates were dissolved in 8 M urea and applied to an RP column. The proteins wild-type L10 and L10Δ10 were separated from L7/L12 by RP HPLC under the same conditions as those used for the other L10 variants. Fractions that contained each protein were pooled and lyophilized.

Preparation of Ribosomes from the Cells Producing Protein L10 Variants. The pellet of crude ribosomes obtained after ultracentrifugation of the S30 extract (see above) was dissolved in buffer A containing 20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 4 mM 2-mercaptoet-

hanol, layered over an equal volume of a 1.1 M sucrose cushion made up in buffer B (buffer A with 0.5 M NH_4Cl), and centrifuged at 100000*g* for 17 h at 4 °C. The pellet was washed gently with buffer A, resuspended in buffer A, dialyzed against the same buffer at 4 °C, and kept in aliquots of 50 μL at −80 °C.

Preparation of Ribosomal Particles Lacking L7/L12 (P_0 Cores). L7/L12 was selectively extracted from 50S subunits as described previously (*28*).

Preparation of Ribosomal Particles Lacking L10 and L7/L12 (P_{37} Cores). L10 and L7/L12 were selectively removed from wild-type 70 S ribosomes by a single extraction with 1 M NH_4Cl and 50% ethanol at 37 °C as described (*29*).

Reconstitution of Particles from P_{37} Cores and L10 Deletion Variants. Quantification of the binding of L10 deletion variants to P_{37} core was determined with the L10 proteins radiolabeled at the single cysteine 70 by modification with [^{14}C]iodoacetamide. The proteins were fully reduced by incubation for 30 min at 37 °C in a solution of 8 M urea with 10 mM dithiothreitol (DTT), then passed through a Bio-Spin gel-filtration column (Bio-Rad) equilibrated in buffer containing 100 mM Tris-HCl, pH 8.3, 4 M urea, and 2 mM DTT. The radiolabeling was carried out in the same buffer containing 7−8 mM [^{14}C]iodoacetamide (8 mCi/mmol) for 2 h at 37 °C. The radiolabeled proteins were separated from excess [^{14}C]iodoacetamide and prepared for reconstitution by gel filtration in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 4 mM 2-mercaptoethanol on a Bio-Spin column. The specific activity of each radiolabeled protein was calculated from liquid scintillation counting (Eco-Lite-(+), ICN Biomedical Inc., Costa Mesa, CA) of the protein sample solution and the protein concentration determined with a Bio-Rad protein microassay standardized against a salt-free, gravimetrically determined sample of wild-type L10.

Ten A_{260} units (230 pmol) of 70S P_{37} cores were incubated with a 2-fold molar excess of one of the radioactive protein L10 variants in 100 μL of reconstitution buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 4 mM 2-mercaptoethanol for 10 min at 37 °C, and then layered over 0.8 mL of a 20% sucrose cushion in the same buffer. The reconstituted particles were recovered by centrifugation at 100 000 rpm for 1.5 h at 4 °C in a TLA-100.2 rotor in a TL-100 ultracentrifuge (Beckman, Palo Alto, CA). Pellets were resuspended in 40 μL of reconstitution buffer, and samples of 4 μL were taken for counting and measuring A_{260}. The recovery of ribosomal particles determined from A_{260} was typically 85%. As a control for nonspecific binding of the proteins to the ribosome, 30S ribosomal subunits were incubated with L10 variants and purified as described above. The amount of L10 variants bound to P_{37} was calculated from the specific activity of the radiolabeled proteins and corrected for the amount of the proteins bound with 30S ribosomal particles.

Quantification of the Binding of L7/L12 to P_{37} Core Particles Reconstituted with L10 Variants. The protein L7/L12Cys-63 was radiolabeled at its single cysteine with [^{14}C]-iodoacetamide using the procedure described for the protein L10 variants, except that the buffers did not contain urea. The concentration of L7/L12Cys-63 was determined with the Bio-Rad protein microassay standardized against wild-type L7/L12. The 70S P_{37} core particles were first incubated with an 2-fold molar excess of each nonlabeled protein L10 variant in reconstitution buffer for 10 min at 37 °C, and then an 8-fold molar excess of radiolabeled L7/L12Cys-63 was added, and incubation was continued for 10 min. As a control, P_{37} cores without L10 were incubated with L7/L12Cys-63. The reconstituted particles were recovered by centrifugation through a sucrose cushion, and the amount of radiolabeled L7/L12Cys-63 bound was determined as described above for L10 variants.

Determination of the Stoichiometry of the Complexes L10−L7/L12 and L10Δ10−L7/L12. The proteins, wild-type L10, L10Δ10, and L7/L12Cys-63, were radiolabeled with [^{14}C]-iodoacetamide as described above. Radiolabeled L7/L12Cys-63 was incubated with labeled wild-type L10 or L10Δ10 in reconstitution buffer for 5 min at 37 °C. The protein mixtures were electrophoresed in a native 8−25% gradient FastGel (Pharmacia Biotech.). The bands containing the stained complexes were cut out and electrophoresed in a 10−20% Tricine/SDS gel (Novex, San Diego, CA). The SDS gel resolved each complex into two components: one corresponding to L7/L12 and the other to wild-type L10 or L10Δ10. The gel was exposed to the Phosphor screen of the STORM system (Molecular Dynamics, Sunnyvale, CA). The amount of radioactivity in each band was quantified with ImageQuant software (Molecular Dynamics). The stoichiometry of the complexes was determined after correction for the specific radioactivity of the labeled proteins.

Native Gel Electrophoresis of Ribosomal Particles. Native 70S ribosomes, 50S subunits, P_{37} and P_0 cores, and reconstituted ribosomal particles were analyzed by native electrophoresis in a 0.5% agarose/3% acrylamide composite gel in buffer containing 25 mM Tris-HCl, pH 8.0, and 1 mM $MgCl_2$ as described previously (*6*). The samples (0.3−0.4 A_{260} unit) were electrophoresed for 16 h, 100 V, at 4 °C with constant recirculation of buffer. The gels were stained with 0.2% methylene blue in 7.5% acetic acid and destained in 1% acetic acid.

EF-G-Dependent GTP Hydrolysis. The assay was carried out as described previously (*30*) with modifications. First, the ribosomal particles were reconstituted by incubation of 70S P_{37} ribosomal cores with wild-type L10 or L10Δ10 and L7/L12 as described above. 70S P_{37} cores, alone and preincubated with L7/L12 without L10, were used as controls. The reaction mixture, in a total volume of 0.25 mL, contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 160 mM NH_4Cl, 12 mM 2-mercaptoethanol, 33 pmol of reconstituted or control particles, and 8 μg of EF-G. The reaction was started by addition of 50 nmol of [γ-^{32}P]GTP (1 Ci/mol) and carried out at 37 °C. Aliquots of 0.05 mL were taken out at times shown in Figure 5, the reaction was terminated, and inorganic phosphate [$^{32}P_i$] released during reaction was counted as described (*30*).

Activity of Ribosomes in Protein Synthesis in Vitro. Ribosomal particles (70S) were reconstituted from 70S P_{37} cores and wild-type L10 or L10Δ10 and L7/L12 as described above. The poly(U)-dependent polyphenylalanine synthesis assay was carried out in a 100 μL reaction mixture containing 30 pmol of reconstituted particles as described previously (*31*). Aliquots of 20 μL were taken out at times shown in Figure 6, the reaction was stopped, and radioactivity bound to ribosomes was counted as described (*32*).

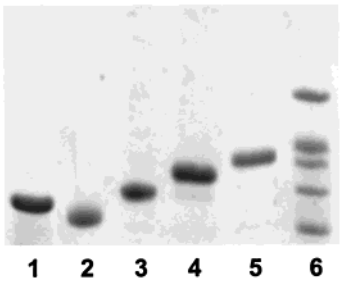

FIGURE 1: SDS−polyacrylamide gel electrophoresis in 20% FastGel of protein L10 variants. Lane 1, L10Δ33; lane 2, L7/L12; lane 3, L10Δ20; lane 4, L10Δ10; lane 5, wild-type L10; lane 6, protein molecular mass markers, 6.5, 14.4, 16.9, 20.4, and 31 kDa.

Table 1: Binding of Radiolabeled Deletion L10 Variants to P_{37} Core Particles

L10 variant added	specific activity (cpm/pmol)	radioactivity bound/A_{260} (cpm)	L10 equiv/particle
L10wt	29.0	669	1.0
L10Δ10	26.5	566	0.9
L10Δ20	25.0	495	0.9
L10Δ33	33.0	737	1.0

RESULTS

Construction of C-Terminal Deletion Variants of the Protein L10. The C-terminal deletions of 10, 20, and 33 amino acids in the gene for the protein L10 were made by PCR mutagenesis as described under Materials and Methods. The genes for the wild-type protein and its truncated variants were coexpressed with the gene for protein L7/L12 in the same genetic construction. Attempts to overproduce L10 alone were unsuccessful due its rapid degradation when it was synthesized in excess over chromosomal L7/L12 (*33*). The deletion mutations and the integrity of the gene for L7/L12 were confirmed by DNA sequencing. The proteins were overproduced, purified, and lyophilized for storage. The proteins were homogeneous and had molecular weights by SDS gel electrophoresis consistent with their predicted amino acid sequences: L10wt, 17 600; L10Δ10, 16 400; L10Δ20, 15 200; L10Δ33, 13 500; and L7/L12, 12 200 (Figure 1). All L10 variants reacted with monoclonal antibodies against wild-type L10 (*34*) (results not shown). For binding experiments, the lyophilized proteins were dissolved in 8 M urea and renatured by gel filtration in reconstitution buffer.

Binding of Deletion Variants of L10 to Ribosomal Core in Vitro. The capacity of the truncated proteins to bind in vitro to ribosomal particles lacking L10 and L7/L12 was tested and quantified by use of the proteins radiolabeled with [^{14}C]iodoacetamide at the single cysteine 70. Wild-type L10 was used in all experiments as a control. Modification of the wild-type L10 with iodoacetamide was shown earlier (*23*) to have no effect on activity of the protein. The results are summarized in Table 1. All truncated proteins were found to bind to the ribosomal particle as efficiently as the wild-type L10, approximately one copy of L10 per ribosome particle.

Binding of L7/L12 to Ribosomal Cores Reconstituted from P_{37} and L10 Variants. To test the ability of L10 variants to bind protein L7/L12 to the ribosome in vitro, the P_{37} core particles were first incubated with the nonlabeled variants of L10 followed by incubation with radiolabeled L7/L12-Cys63 as described under Materials and Methods. L7/L12-Cys63 was chosen because it could be easily and quantitatively radiolabeled with [^{14}C]iodoacetamide at the single cysteine 63, and it had previously been shown to be fully active in protein synthesis, and the modification with iodoacetamide had no effect on its binding (*28*). The reconstituted particles were recovered by centrifugation through a sucrose cushion. The results are presented in Table 2. The particles reconstituted with wild-type L10 gave 3.1 copies of bound L7/L12, a value that is in agreement with those typically obtained (3−4 copies of L7/L12) in in vitro reconstitution experiments (*35*). Experiment 1 shows the binding of L7/L12 to the particles made with L10Δ10 to be half that for the P_{37} preincubated with wild-type L10. The small amount of radioactivity bound to the P_{37} particles reconstituted with either L10Δ20 or L10Δ33 (experiments 2 and 3) was nearly the same as that bound to P_{37} cores lacking all of L10. This indicates that these two L10 variants do not bind any L7/L12. The most plausible explanation of these results is that L10Δ10 binds only one dimer of L7/L12 and that one-dimer ribosomal particles were formed.

Table 2: Binding of Radiolabeled L7/L12-Cys63[a] to P_{37} Core Particles Reconstituted with L10wt or L10Δ10

expt	reconstituted particles	specific activity (cpm/pmol)	radioactivity bound/A_{260} (cpm)	copies of L7/L12/particle
1	P_{37} + L10wt + L7/L12	19.0	1429	3.3
	P_{37} + L10Δ10 + L7/L12	19.0	739	1.7
	P_{37} + L7/L12	19.0	93	0.2
2	P_{37} + L10wt + L7/L12	21.7	1718	3.5
	P_{37} + L10Δ20 + L7/L12	21.7	134	0.3
	P_{37} + L7/L12	21.7	182	0.4
3	P_{37} + L10wt + L7/L12	170	13 396	3.4
	P_{37} + L10Δ33 + L7/L12	170	2573	0.6
	P_{37} + L7/L12	170	1575	0.4

[a] In experiments 1 and 2, L7/L12-Cys63 was labeled with [^{14}C]iodoacetamide as described under Materials and Methods, and in experiment 3, the protein was labeled with ^{125}I-APDP as described (*36*).

Experiments to examine the possible heterogeneity of the reconstituted material were carried out to exclude the possibility that half of the ribosomes with L10Δ10 had two dimers and half had no dimers of L7/L12. Reconstituted particles were analyzed by native electrophoresis in an agarose/acrylamide gel at a low concentration of Mg^{2+} to dissociate 70S particles into 50S and 30S subunits (Figure 2). The gel was shown earlier (*6*) to resolve 50S particles, 50S P_0 cores lacking completely L7/L12, and 50S P_{37} cores lacking both L7/L12 and L10. The 30S subunits appear on the gel as diffuse bands toward the bottom of the gel. It is seen clearly on the gel that reconstitution of P_{37} cores (minus both L7/L12 and L10) (lane 1) with either wild-type L10 (lane 3) or L10Δ10 (lane 4) resulted in formation of particles with mobility similar to that of P_0 cores (minus only L7/L12) (lane 2). The particles reconstituted as P_{37} + L10wt + L7/L12 (lane 6) have the same mobility as untreated 50S subunits, indicating that two dimers of L7/L12 are bound. Reconstitution of P_{37} with L10Δ10 and L7/L12 led to the formation of a single new type of particle (lane 5) that had intermediate mobility between P_0 cores (lanes 2−4) and 50S subunits (lane 7) or fully reconstituted particles made with the wild-type L10 (lane 6). The population of the particles with L10Δ10 that binds half as much L7/L12 as full-length L10 is homogeneous by electrophoresis. There are no

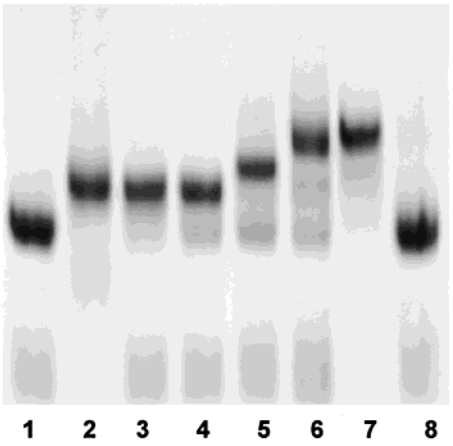

FIGURE 2: Native electrophoresis in 0.5% agarose/3% acrylamide gel of reconstituted ribosomal particles. 70S P_{37} ribosomal core particles, from which the wild-type proteins L10 and L7/L12 were selectively and completely removed, were incubated with wild-type L10 or L10Δ10 followed by incubation with L7/L12. Lane 1, 70S P_{37} core particles; lane 2, 50S P_0 core particles prepared as described under Materials and Methods; lane 3, 70S P_{37} + L10wt; lane 4, 70S P_{37} + L10Δ10; lane 5, 70S P_{37} + L10Δ10 + L7/L12; lane 6, 70S P_{37} + L10wt + L7/L12; lane 7, 50S subunits; lane 8, 70S P_{37} + L7/L12.

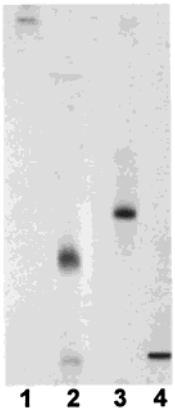

FIGURE 3: Native electrophoresis in 8−25% gradient FastGel of L10wt-L7/L12 and L10Δ10-L7/L12 complexes. The complexes were formed by mixing solutions of the pure proteins followed by incubation for 5 min at 37 °C. Lane 1, wild-type L10; lane 2, complex L10Δ10−L7/L12; lane 3, complex L10wt−L7/L12; lane 4, L7/L12.

particles that correspond to the two-dimer particles. Crude reconstitution mixtures before the centrifugation step were also analyzed by native gel electrophoresis under the same conditions. The gel patterns were the same as that for reconstituted particles purified by high-speed centrifugation. This implies that the new particle containing a single L7/L12 dimer was stable to high-speed centrifugation.

Formation of the L10Δ10−L7/L12 Complex in Solution and Its Stoichiometry. The capacity of protein L10Δ10 to interact with L7/L12 in solution, in the absence of ribosomal particles, to form a trimer, L10Δ10·$(L7/L12)_2$, instead of the wild-type pentamer, L10·$(L7/L12)_4$, was tested. The pure L10Δ10 was mixed with L7/L12 in ratios from 1:1 to 1:6 and incubated for 5 min at 37 °C. As a control, the same was done with wild-type L10 and L7/L12. The mixtures were analyzed by native electrophoresis in an 8−25% acrylamide gel (Figure 3). Neither protein L10Δ10 nor wild-type L10 enter the gel under the electrophoresis conditions. Both

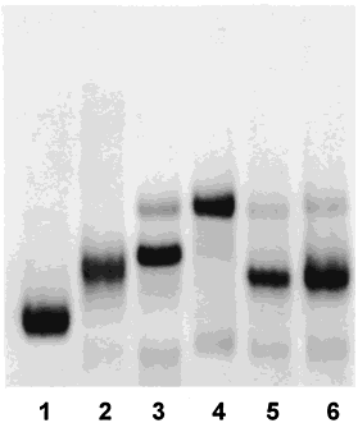

FIGURE 4: Native electrophoresis in 0.5% agarose/3% acrylamide gel of the ribosomes purified from the cells overproducing L10 variant proteins**.** Ribosomes were purified from cells harvested 3−5 h after adding IPTG. 70S P_{37} and 50S P_0 core particles were prepared as described under Materials and Methods. Lane 1, 70S P_{37} core particles; lane 2, 50S P_0 core particles; lane 3, ribosomes purified from the cells producing L10Δ10; lane 4, wild-type ribosomes; lanes 5 and 6, ribosomes purified from the cells producing L10Δ20 and L10Δ33, respectively.

proteins when mixed with varying amounts of the acidic protein L7/L12 formed a single new component or complex that migrated into the gel. The mobility of the putative L10Δ10−L7/L12 complex was different from the mobility of L10wt−L7/L12, L8, complex. The bands of complexes were cut out and electrophoresed a second time in an SDS gel. Each complex gave two bands: one corresponding to L7/L12 and the other to wild-type L10 or L10Δ10 (data not shown).

The stoichiometry of the complexes was determined by use of proteins L10, L10Δ10, and L7/L12-Cys63 radiolabeled with [^{14}C]iodoacetamide. Bands were cut out from the native gel and resolved into the components by SDS gel electrophoresis, and the gel was exposed to the Phosphor screen of the STORM system. The amount of radioactivity in each protein band was quantified. After correction for the specific radioactivity of the proteins, the stoichiometry of the L10wt−L7/L12 and L10Δ10−L7/L12 complexes was determined. The molar ratio of L7/L12 to L10wt was 4, consistent with the stoichiometry of L8 complex (*5*, *7*). The ratio of L7/L12 to L10Δ10 was 2, indicating that L10Δ10 bound only two copies of L7/L12 or one dimer. Incubation of L10Δ20 or L10Δ33 with L7/L12 and subsequent analysis of the mixtures by native gel electrophoresis did not show the formation of any protein complexes.

Occurrence of One L7/L12 Dimer Ribosomal Particles in Vivo. Ribosomes were isolated and purified from cells producing L10Δ10 as described under Materials and Methods. The ribosomes were analyzed by native electrophoresis in an agarose/acrylamide gel. The results in Figure 4 show that at the moment of harvesting cells (5 h after inducing the production of L10Δ10) the population of ribosomes in the cells (lane 3) contained approximately 20% ribosomes with two L7/L12 dimers and 80% particles with a single dimer. The population of the ribosomes purified from the cells producing proteins L10Δ20 (lane 5) and L10Δ33 (lane 6) contained two-dimer ribosomes and particles with no L7/L12 but no intermediate particles. This confirms the in vitro results showing the absence of L7/L12 binding to the two

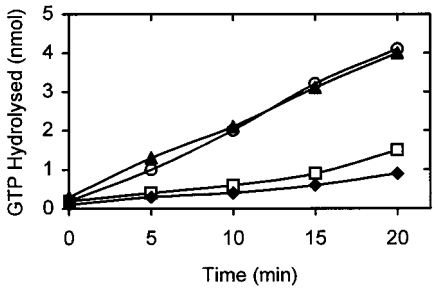

FIGURE 5: Kinetics of EF-G dependent GTPase of reconstituted ribosomal particles. (◆) 70S P_{37} core particles; (□) 70S P_{37} cores incubated with L7/L12 without L10; (○) ribosomal particles reconstituted from 70S P_{37} cores with L10Δ10 and L7/L12; (▲) ribosomal particles reconstituted from 70S P_{37} cores with L10wt and L7/L12.

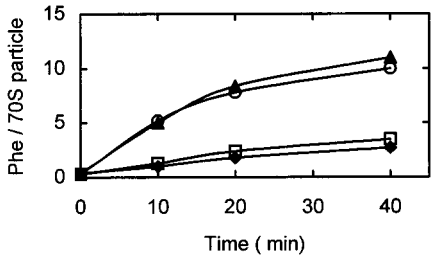

FIGURE 6: Kinetics of poly(U)-dependent polyphenylalanine synthesis promoted by reconstituted ribosomal particles. The assay was carried out as described under Materials and Methods. Symbols used are the same as in Figure 5.

L10 variants with longer deletions. The ratio of the fraction of two-dimer ribosomes to that of either one-dimer or "no L7/L12" particles shown above was in rough agreement with the ratio of the amount of wild-type L10 to that of corresponding truncated L10 protein variant present in the whole population of the ribosomes, determined by immunoblot analysis of the ribosomes (data not shown).

Activity of Ribosomal Particles with One L7/L12 Dimer in Vitro. 70S P_{37} core particles were reconstituted with L10Δ10 and L7/L12 to yield particles containing a single L7/L12 dimer as described under Materials and Methods, and reconstitution was also done with wild-type L10. As a control, L7/L12 was incubated with P_{37} alone. Two assays were carried out to test the activity of reconstituted particles in vitro: EF-G-dependent GTP hydrolysis and poly(U)-dependent polyphenylalanine synthesis. The results presented in Figures 5 and 6, respectively, shows that the particles with one L7/L12 dimer support EF-G-dependent GTP hydrolysis and protein synthesis in vitro to the same extent and at the same rate over the extended time period measured as two-dimer reconstituted ribosomes.

DISCUSSION

The main goal of this research was to construct ribosomal particles that contain only one of the two L7/L12 dimers in order to further our understanding of the location, conformation, dynamics, and function of each of the dimers individually. Protein engineering of L10 was chosen as the strategy for creating the single-dimer particles. We wanted first to determine the sites in the L10 sequence that are necessary for binding each L7/L12 dimer and then, keeping the binding site for one dimer intact, to destroy by deletion or inactivation the second binding site. The three-dimensional structure of the protein L10 is not known at present. Protein L10 consists of 164 amino acids with a single cysteine as residue 70 (*24*). Chemical cleavage at this residue permitted investigation of the functional properties of each resulting fragment. The N-terminal fragment (residues 1−69) is important for binding L10−$(L7/L12)_4$ complex to the ribosome through interaction with 23S RNA in the 50S ribosomal subunit (*15*). The C-terminal fragment (residues 71−164) binds both L7/L12 dimers (*23*). On the basis of these results, we narrowed the search of the binding sites for L7/L12 on L10 to the residues 71−164. Protein L10 variants with C-terminal deletions of 10, 20, and 33 amino acids have been obtained. The deletions in the C-terminal portion of the protein did not significantly perturb the structure of the ribosome binding domain in the N-terminal part.

Native electrophoresis in acrylamide gel was chosen as a suitable method for monitoring the interaction of L10 variants with L7/L12 in solution (in the absence of ribosome). The electrophoresis allowed each of the protein complexes formed to be well separated from the other components of the protein mixture and thereby facilitated determination of the stoichiometry of the complexes. Protein L10Δ10 bound specifically only one L7/L12 dimer. No pentameric complex was formed in the mixture of L10Δ10 and L7/L12 if the ratio of L7/L12 to L10Δ10 was 4:1 and greater. The binding of two L7/L12 dimers to wild-type L10 seems to be cooperative as no formation of any single-dimer intermediate was observed in the mixture of wild-type L10 and limited amount of L7/L12 (when the ratio of L7/L12 to L10 was 3:1 or 2:1 instead of 4:1) (results not shown).

L7/L12 has been previously shown to be required for a number of partial reactions in protein synthesis dependent on the presence of elongation factors and GTP hydrolysis (*12*). The complete extraction of L7/L12 (all four copies) led the ribosomal particles to lose most of their capability to promote these reactions (*13*). Two laboratory groups studied factor-dependent GTPase activity of reconstituted ribosomal particles and their activity in poly(U)-dependent polyphenylalanine synthesis as a function of the amount of L7/L12 added to depleted core particles (*32*, *35*). Both groups concluded that the binding of one dimer was sufficient for full EF-G-dependent GTPase activity. They disagreed concerning whether full activity in polyphenylalanine synthesis was achieved with one or two dimers. It was also unclear which of two dimers participated in those reactions. Our result shows directly that the one particular dimer described here is sufficient for full ribosome activity in both in vitro tests. The question of the functional role of the second L7/L12 dimer is still to be answered.

We have constructed one of two possible ribosomal particles that contain only one L7/L12 dimer. The presence of the single-dimer particles was also shown in vivo. Our finding that L10 lacking 20 C-terminal amino acids (145−164) binds no L7/L12, and the protein with 10 deleted C-terminal residues (155−164) binds one L7/L12 dimer, implies that some or all of the residues 145−154 are involved in interaction with one ("internal-site") dimer (the one retained and reported here) and that residues 155−164 determine the binding site for the second ("C-terminal-site") dimer. The question of whether the sequences implicated are simple linear binding determinants or are involved in the constitution of a higher order binding sites remains open.

The particle containing a single L7/L12 dimer presents an ideal system for studying the location, conformation, dynamics, and function of the "internal-site" L7/L12 dimer: its susceptibility to removal by monoclonal antibodies; whether it appears as a "stalk" by immuno and cryo-electron microscopy; its pattern of cross-linking to near and distant proteins; its motions and dynamics determined by fluorescence and NMR. Localization of the "internal binding site" within residues 145−154 points to a strategy for creating the second type of single-dimer particle containing L7/L12 bound only to "C-terminal-site" and investigating similarly the properties of the second dimer.

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