A Point Mutation in Ribosomal Protein L7/L12 Reduces Its Ability to Form a Compact Dimer Structure and to Assemble into the GTPase Center†

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ABSTRACT: An *Escherichia coli* mutant, LL103, harboring a mutation (Ser15 to Phe) in ribosomal protein L7/L12 was isolated among revertants of a streptomycin-dependent strain. In the crystal structure of the L7/L12 dimer, residue 15 within the N-terminal domain contacts the C-terminal domain of the partner monomer. We tested effects of the mutation on molecular assembly by biochemical approaches. Gel electrophoretic analysis showed that the Phe15-L7/L12 variant had reduced ability in binding to L10, an effect enhanced in the presence of 0.05% of nonionic detergent. Mobility of Phe15-L7/L12 on gel containing the detergent was very low compared to the wild-type proteins, presumably because of an extended structural state of the mutant L7/L12. Ribosomes isolated from LL103 cells contained a reduced amount of L7/L12 and showed low levels (15−30% of wild-type ribosomes) of activities dependent on elongation factors and in translation of natural mRNA. The ribosomal activity was completely recovered by addition of an excess amount of Phe15-L7/L12 to the ribosomes, suggesting that the mutant L7/L12 exerts normal functions when bound on the ribosome. The interaction of Ser15 with the C-terminal domain of the partner molecule seems to contribute to formation of the compact dimer structure and its efficient assembly into the ribosomal GTPase center. We propose a model relating compact and elongated forms of L7/L12 dimers. Phe15-L7/L12 provides a new tool for studying the functional structure of the homodimer.

Ribosomal protein L7/L12 is the only protein present in more than a single copy per *Escherichia coli* ribosome. The structural state of L7/L12 and its function dependent on translation factors in the ribosome have long been investigated by biochemical approaches (*1*−*3*), NMR spectroscopy (*4*−*10*), and cryo-electron microscopy (*11*−*14*). L7/L12 is present in four copies as two dimers on the 50S subunit (*15*−*19*). Each L7/L12 monomer is divided into a globular C-terminal domain (CTD)[1] and an N-terminal domain (NTD), responsible for its dimerization (*20*−*23*). These two dimers of L7/L12 are anchored to another protein, L10 (*24*−*26*). The L10-L7/L12 complex binds to a unique region, termed the thiostrepton domain, around position 1070 of 23S rRNA through protein L10 (*27*−*29*), and constitutes a part of the ribosomal GTPase-associated center. Considering a role of L7/L12 in the stimulation of factor-dependent GTPase activity (*30*, *31*) and its flexible nature (*6*−*8*, *11*−*14*, *32*−*36*), this protein appears to participate in the dynamic mechanism of protein synthesis.

Protein engineering studies have shown that the intact form of the pentameric structure of L10-L7/L12 complex is not essential for ribosome function, i.e., either removal of a single L7/L12 dimer from the anchor protein L10 (*37*) or deletion of one, although not two, CTD from each L7/L12 dimer (*38*) gives no marked effect on the ribosomal activity in translation elongation. Ribosomes reconstituted with dimers cross-linked in different orientations of the CTDs were also active (*39*, *40*). However, the dimerization of L7/L12 seems to be crucially important for functional incorporation into the ribosome. This view was first suggested by Gudkov's group (*21*). They obtained a modified L7/L12 that failed to form the dimer and assemble to the ribosome by exposure of L7/L12 to oxidative conditions containing 0.3 M H_2O_2 at pH 3.2 (*21*, *22*). Molecular details of L7/L12 dimerization and its functional significance are still not fully understood.

Current X-ray crystallographic analysis has failed to resolve the fine structural state of L7/L12 within the 50S subunit (*41*). The same technique has been successfully used for the isolated L7/L12 proteins; Leijonmarck and Liljas have shown structure of the C-terminal domain of *E. coli* L7/L12 (*42*), and Wahl et al. have recently solved the structure of entire L7/L12 molecules from *Thermotoga maritima* (*43*). Wahl et al. proposed two alternative dimerization modes. In mode I, the two L7/L12 monomers form a tight, symmetric, and parallel dimer mainly through a four-helix bundle of the

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[1] Abbreviations: CTD, carboxyl terminal domain; NTD, amino terminal domain; SDS, sodium dodecyl sulfate; EF-Tu, elongation factor Tu; EF-G, elongation factor G.

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N-terminal domains. In mode II, the N-terminal domains of the two monomers bind to each other in different orientation from mode I, so that the two CTDs are located in distant positions. Both models are different from those proposed previously (*3*, *33*, *44*); a major new and unexpected point in mode I is the presence of additional interactions between the NTD of one monomer and the CTD of the partner molecule. The NTD−CTD contacts in mode I result in a rather compact instead of elongated property of the dimer. This is in disagreement with the flexible nature of the CTD observed in the ribosome. It is important to test the view of L7/L12 dimerization from the crystal data by biochemical approaches. We report here on a point mutation of L7/L12 at the position Ser15, a residue involved in the NTD−CTD interaction in mode I dimerization based on the crystal structure (*43*). This mutant (LL103) was isolated as a spontaneous revertant of streptomycin dependence, suggesting that this mutation affects the processes at the decoding center of 30S subunits as well as at the GTPase-associated center. We show that the Ser15 to Phe mutation of L7/L12 changes the structural features and causes reduced ability of its incorporation into the GTPase-associated center. We also show that Phe15-L7/L12 (LL103-type) functions comparably to wild-type L7/L12, when the ribosomes are fully loaded with the mutant dimers by adding them in excess. We discuss the significance of the NTD−CTD interaction in assembly and stability of the GTPase-associated center.

MATERIALS AND METHODS

Isolation of the Ribosomal Mutant Strain LL103. Streptomycin independent revertant LL103 was produced spontaneously from streptomycin-dependent strain VT, and isolated as described previously (*45*).

Ribosomes, the 50 S Core Particles, and the Reconstitution of 50 S Subunits. E. coli strains LL103 and Q13 (as a source of the wild-type ribosomes). *E. coli* cells were grown in LB medium and harvested at the exponential stage. Salt-washed ribosomes (*46*) and the 50S and 30S subunits (*47*) were prepared as described. The 50S core particles deficient in L7/L12 were prepared in 50% ethanol/0.5 M NH_4Cl according to Hamel et al. (*30*). The core 50S particles deficient in both the L10.L7/L12 complex and L11 were prepared by using L11-deficient *E. coli* ribosomes (*48*), as previously described (*47*). Extraction of the specific proteins L7/L12 or L10-L7/L12 was confirmed by 16.5% SDS−polyacrylamide gel electrophoresis (*49*). The reconstitution of 50S subunits from cores deficient in L7/L12 or in L10-L7/L12 and L11 was performed by adding excess amounts of isolated proteins in buffer conditions described in figure legends.

Sequencing of Gene for L7/L12 (rplL). Genomic DNA of *E. coli* cells was prepared according to Smith et al. (*50*). DNA fragments covering entire coding sequence of L7/L12 (*rplL*) were amplified by the polymerase chain reaction (PCR, ref *51*) using two oligonucleotide primers for the flanking sequences, 5′-ATTCTGATATTCAGGAACAATTTAA-3′ and 5′-ATTTCTCAGGCTGCAACCGGAAG-3′. The DNA fragments were inserted into plasmid pT7Blue (Novagen) and sequenced by dideoxy method (*52*). The mutation within LL103 *rplL* was verified by three independent experiments.

Plasmid Construction, Protein Overexpression, and Purification. The L7/L12 coding regions in LL103 and Q13 cells were amplified by PCR using primers: (a) 5′-GAGCGAGCGACATATGTCTATCACTAAAGA-3′ containing the start codon as well as a *Nde*I site and (b) 5′-GCGGGATCCTTATTTAACTTCAACTTCAGC-3′ containing the sequence corresponding to the stop codon and *Bam*HI site. The DNA fragments were inserted within *Nde*I/*Bam*HI sites of an expression vector pET3a (Novagen). To express L10, we amplified DNA encompassing the L10 gene (*rplJ*) linked with L7/L12 gene (*rplL*) by PCR using genome DNA from Q13 and primers: 5′-GAGAGAGACATATGGCTTTAAATCTTCAAG-3′ and primer b). The DNA fragment was cloned into *Nde*I/*Bam*HI sites of pET3a. Coexpression of L10 and L7/L12 was essential for L10 expression, as described by Griazova et al. (*37*). The plasmid for expression of L10Δ10 variant lacking 10 amino acid residues at the C-terminus (*37*) was a kind gift from Drs. R. R. Traut and O. Griaznova, University of California, Davis. Proteins were expressed in *E. coli* BL21 (DE3) pLysS (Novagen) as described by Griaznova and Traut (*37*).

The S100 soluble fraction was dialyzed overnight against buffer A containing 20 mM sodium acetate pH 4.4, 7 M urea, and 5 mM 2-mercaptoethanol, and then applied to a column of CM-cellulose (Whatman) equilibrated with the same buffer. L7/L12 protein was eluted with buffer A. L10 or L10Δ10 was eluted in the same buffer containing 0.075 M of LiCl. The L7/L12 fraction was dialyzed overnight against buffer B containing 20 mM sodium phosphate pH 6.5, 6 M urea, 20 mM LiCl, 1 mM DTT and further purified by high-performance ion-exchange chromatography (HPLC) with DEAE-5PW (Tosoh) in a linear gradient of 20−265 mM LiCl. The L10 or L10Δ10 fraction was dialyzed to buffer C containing 20 mM sodium phosphate pH 6.5, 6 M urea, 20 mM LiCl, 5 mM 2-mercaptoethanol and purified by HPLC with CM-5PW (Tosoh) in a linear gradient in 20−216 mM LiCl. Purity of the proteins was confirmed by SDS−polyacrylamide gel electrophoresis. All the proteins were dialyzed overnight against a buffer containing 10 mM HEPES−KOH pH7.5, 300 mM KCl, 10 mM 2-mercaptoethanol, and stored at −80 °C.

Analysis of Protein−Protein Binding by Gel Electrophoresis. Isolated L7/L12 and L10 samples and their mixture were incubated in 10 μL of solution at 37 °C for 5 min, as described in the figure legend. Electrophoresis was performed using 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 39:1) for 10 h at 100 V and 4 °C with buffer recirculation. Running buffer contains 100 mM KCl and 20 mM Tris-HCl pH 8.7. The same gel analysis was performed in the presence of 0.05% nonionic detergent Triton X-100.

Ribosome Functional Assays. The EF-G-dependent GTPase activity was carried out as described previously (*46*), except that reaction mixture contained 2.5 pmol of 50S subunits, 5 pmol of wild-type 30S subunits and 7 mM $MgCl_2$. EF-Tu/EF-G-dependent polyphenylalanine synthesis was performed as described (*47*), except that the reaction mixture contained 10 pmol of 50S subunits and 20 pmol of wild-type 30S subunits. Ribosomal activity in translation of the dihydrofolate reductase (DHFR) gene was also assayed by using an in vitro transcription−translation coupled system that is composed of all required isolated factors (termed the PURE System), according to Shimizu et al. (*53*) with some modifications, viz., (i) our reaction mixture (30 μL) contained 20 pmol of 50S subunit samples, 40 pmol of wild-type 30S

subunits, 153 mM potassium glutamate, 18.7 mM potassium acetate, 2 mM spermidine, 20 mM creatine phosphate and 0.34 A_{260} units tRNA mix (Roche); (ii) we removed 5 mM potassium phosphate, 5 mM NH_4Cl, 0.5 mM $CaCl_2$, and 8 mM putrescine from the reaction mixture. The amounts of translation products were estimated by radioactivity of incorporated [^{35}S]Met into DHFR and its enzyme activity (*54*).

Quantitative Analysis of L7/L12 in the 50S Subunits. The ribosomal proteins of 50S subunits from LL103 and Q13 strains (0.43 A_{260} unit each), together with various amounts of isolated L7/L12, were subjected to 16.5% SDS−polyacrylamide gel electrophoresis (*49*). The gel was stained with fluorescent regent, SYPRO Orange (Molecular Probes). Intensity of clearly resolved bands for L7/L12 was measured with STORM 860 PhosphorImager (Molecular Dynamics) using ImageQuant software and compared with that of standard L7/L12 samples.

Electrophoresis of 50S Subunits with Acrylamide/Agarose Composite Gel. The 50S subunits and reconstituted particles (0.3 A_{260} unit each) were analyzed by electrophoresis on acrylamide/agarose composite gel composed of 3% acrylamide and 0.5% agarose (*55*), as described previously (*47*).

RESULTS

Properties of 50S Ribosomal Subunits from E. coli LL103 Mutant. E. coli strain VT is a streptomycin-dependent mutant that has been used to select spontaneous revertants to streptomycin independence. Many of these revertants harbor mutations in a number of different ribosomal proteins (*45*). Among them, four mutations of L7/L12 have been characterized previously (*56*, *57*). Using this approach, we obtained a novel L7/L12 mutation revertant, LL103. This strain grew with a 2-times longer doubling time than the Q 13 strain. L7/L12 and the L10-L7/L12 complex (L8) from LL103 ribosomes were not detected by two-dimensional urea gel electrophoresis, although all other 50 S ribosomal proteins were present in normal amounts (data not shown). SDS gel electrophoresis also showed no marked difference in 50 S ribosomal proteins between the wild-type (Figure 1A, lane 1) and LL103 (lane 3), except that LL103 ribosomes contain only a small amount of L7/L12. This protein was selectively extracted in 50% ethanol from 50S subunits of LL103 (lane 4) as well as the wild-type (lane 2). The L7/L12 protein extracted from LL103 ribosomes showed the same mobility as the wild-type protein (lane 5). The gel was stained with the fluorescent dye SYPRO Orange and band intensity for LL103 L7/L12 was compared with that of the wild-type. The fluorescence intensity for LL103 L7/L12 was 27−29% of wild-type L7/L12 in three independent experiments. The amount of L7/L12 recovered by ethanol extraction of LL103 50S subunits was 25−28% of wild-type 50S subunits. The results indicate that LL103 50S subunits contain significantly reduced amounts of L7/L12.

The activity of LL103 50S ribosomal subunits was assayed in vitro with wild-type 30S subunits. As expected from the reduced content of L7/L12, LL103 50S subunits showed a low level, about 15% of wild-type 50S subunits, of EF-G-dependent GTPase activity (Figure 1B) that was nonetheless higher than that of the L7/L12-deficient core particles prepared by the ethanol treatment. A similar level of activity

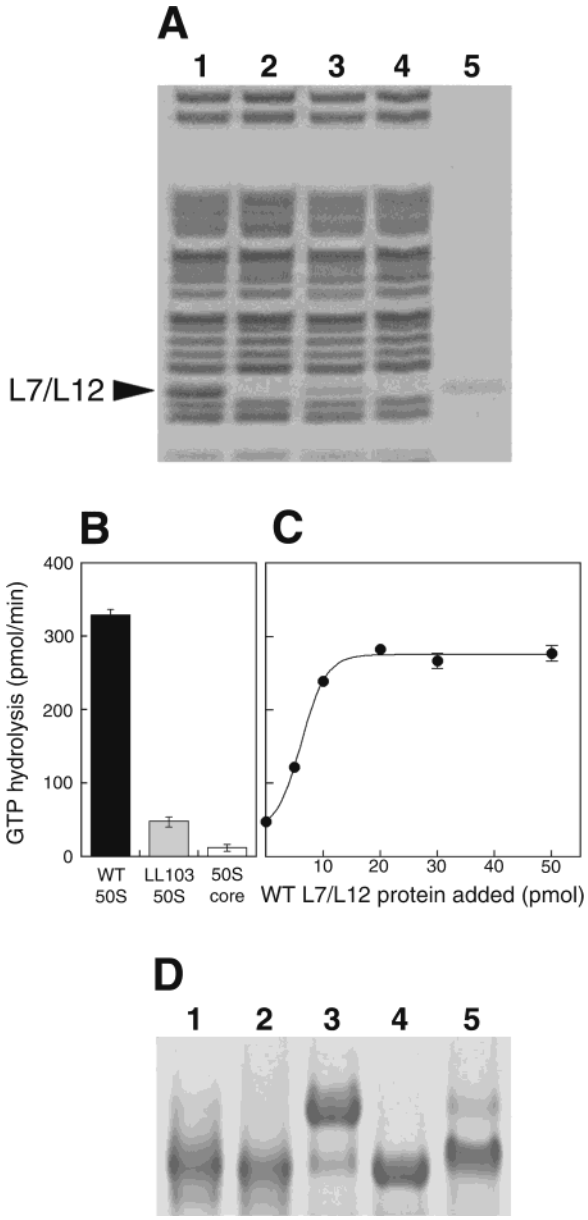

FIGURE 1: Characterization of isolated 50S subunits from LL103 strain. (A) The ribosomal protein from 0.43 A_{260} unit of isolated 50S subunits from Q13 (lane 1) and LL103 (lane 3) strains, and the respective particles treated in 50% ethanol/0.5 M NH_4Cl to remove L7/L12 (lanes 2 and 4), together with 0.1 μg of LL103 L7/L12 (lane 5), were analyzed by 16.5% SDS−polyacrylamide gel electrophoresis. The gel was stained with fluorescent regent, SYPRO Orange, and amounts of L7/L12 in the 50S subunits were estimated, as described in Materials and Methods. A band corresponding to wild-type L7/L12 is marked. (B) Each 50S subunit sample supplemented with wild-type 30S subunits was tested for EF-G-dependent GTPase activity. The assays were carried out by incubation at 37 °C for 10 min in buffer containing 7 mM $MgCl_2$, 50 mM NH_4Cl, 20 mM Tris-HCl pH 7.6, and 5 mM 2-mercaptoethanol. (C) LL103 50S subunits was preincubated with increasing amounts of wild-type L7/L12 and then tested for EF-G-dependent GTPase activity with the supplemented wild-type 30S subunits. (D) Each 50S subunit sample (0.3 A_{260} unit) was analyzed by agarose/acrylamide composite gel electrophoresis: lane 1, LL103 50S subunit; lane 2, core particle of LL103 50S subunit; lane 3, Q13 (wild-type) containing two L7/L12 dimers; and lane 4, core particle of wild-type 50S subunit; lane 5, particle containing a single L7/L12-dimer reconstituted by incubation of the L7/L12·L10 and L11-deficient core particle with L10Δ10 (37), L11 and wild-type L7/L12 in 1 mM $MgCl_2$, 50 mM NH_4Cl, and 25 mM Tris-HCl, pH 7.6.

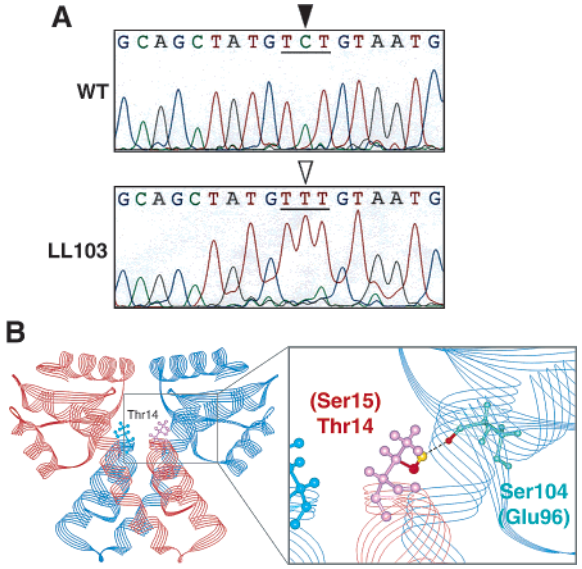

FIGURE 2: Position of the point mutation in LL103 L7/L12. (A) The partial base sequences of the L7/L12 genes from Q13 (wild-type) (upper panel) and LL103 (lower panel) strains were indicated with the wave pattern. Wave colors are represented in blue, guanine; black, adenine; red, thymine and green, cytosine. The underlined codon, TCT in wild-type and TTT in LL103 encode serine and phenylalanine, respectively. (B) The position of the point mutation is shown in crystal structure of L7/L12 dimer (mode I) from *Thermotoga maritima* (PDB code 1DD3, ref *43*). One monomer is represented in red, the other in blue. Thr14 in *T. maritma* corresponds to Ser15 in *E. coli*. Oxygen (red) and hydrogen (yellow) involving in a hydrogen bond between hydroxyl group of Thr14 and carbonyl group of Ser104 are colored. The structural models were produced by programs, WebLab viewerLite 3.2 and Swiss PDB Viewer 3.7b2.

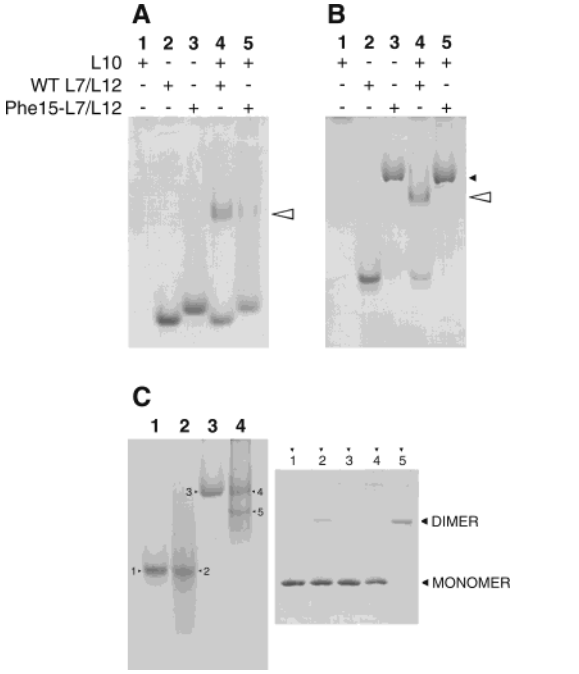

FIGURE 3: Gel electrophoretic analysis of L7/L12 dimer and the L10-L7/L12 complex. The ribosomal protein samples were analyzed by 6% acrylamide gel electrophoresis in the absence (A) and presence of 0.05% Triton X-100 (B): lane 1, 76 pmol of L10; lanes 2 and 3, 460 pmol of wild-type and Phe15-L7/L12, respectively; lane 4, 76 pmol L10 + 460 pmol wild-type L7/L12; lane 5, 76 pmol L10 + 460 pmol Phe15-L7/12. The gels were stained with Coomassie Brilliant Blue. Open arrowheads indicate the positions corresponding to L10-L7/L12 complex. (C, left) mobility of 460 pmol of wild-type L7/L12 (lane 1) and Phe15-L7/L12 (lane 3) in the same Triton X-100 gel as B was compared with respective proteins (920 pmol each) treated with 1 mM glutaraldehyde for 10 min at 25 °C (lanes 2 and 4). Protein bands numbered on the gel were cut out and subjected to SDS−polyacryoamide gel electrophoresis (C, right). Protein samples for lanes 1−5 of the right panel correspond to bands numberd on the left gel, respectively. Marks DIMER and MONOMER correspond to the positions for cross-linked dimers and non-cross-linked monomers, respectively.

of LL103 50S subunits was also observed in poly(U)-dependent polyphenylalanine synthesis (data not shown). To confirm that this reduced ribosomal activity was due to only alteration of L7/L12, we tested the effect of addition of the wild-type L7/L12 protein to LL103 50S subunits. The EF-G-dependent GTPase activity of LL103 ribosomes was recovered up to the level of wild-type ribosomes by an addition of wild-type L7/L12 (Figure 1C).

We performed acrylamide/agarose composite gel electrophoresis (Figure 1D) that has been used to resolve 50S core particles lacking L7/L12 from particles with two or more copies of L7/L12 (*55*). The intact 50S subunits containing four copies of L7/L12 showed low mobility (lane 3) compared to the core particles completely lacking L7/L12 (lane 4) and the particles containing a single dimer (two copies) of L7/L12 (lane 5) that was reconstituted using the C-terminal deletion mutant of L10 protein (L10Δ10), as described by Griaznova et al. (*37*). As shown in lane 1, most of LL103 50S subunits migrated as fast as the core particles completely deficient in L7/L12 (lane 2). The smearing of this lane can be explained by a heterogeneity of LL103 50 S subunits. There seems to be a small group of particles carrying a single dimer of L7/L12 and a minor group of the 50S subunits containing four copies of L7/L12.

The Ser15 to Phe Mutation in L7/L12 of LL103 Strain. The base sequence of the L7/L12 gene from LL103 strain showed only a point mutation, C to T, causing alteration of Ser15 to Phe (Figure 2A), whereas no change was detected in the gene for L10 to which L7/L12 binds (not shown). The amino acid, Ser or Thr, at position 15 (*E. coli* numbering) is conserved in most bacteria. The location of Thr14 (corresponding to position 15 of *E. coli* L7/L12) in *T. maritima* L7/L12 can be seen in crystal structures reported by Wahl et al. (*43*). Thr14 lies in a turn loop between helices α1 and α2 of the NTD. When the L7/L12 dimer is formed in mode I (Figure 2B), this amino acid residue of one monomer interacts with a part of the backbone structure (carbonyl oxygen) of Ser104 in the CTD of the other monomer by a hydrogen bond (Figure 2B). The same hydrogen bond is feasible when Thr of this position is replaced with Ser. Therefore, it is likely that Ser15 in *E. coli* L7/L12 monomer is involved in interaction with the partner molecule through the NTD−CTD hydrogen bond.

Characterization of the Phe15 Variant of L7/L12. The DNA fragments encoding wild-type L7/L12 and its Phe15 variant were cloned into the pET3a protein expression vector. Proteins, expressed in *E. coli* cells and purified, were subjected to native gel electrophoresis (Figure 3A). Compared to wild-type L7/L12 (lane 2), Phe15-L7/L12 showed slightly lower mobility with some smearing (lane 3). A new band appeared when L10 was added to wild-type L7/L12 (lane 4), although L10 alone failed to enter the gel (lane 1). By contrast, addition of L10 to Phe15-L7/L12 gave only a weak signal of the complex formation (lane 5). To see more

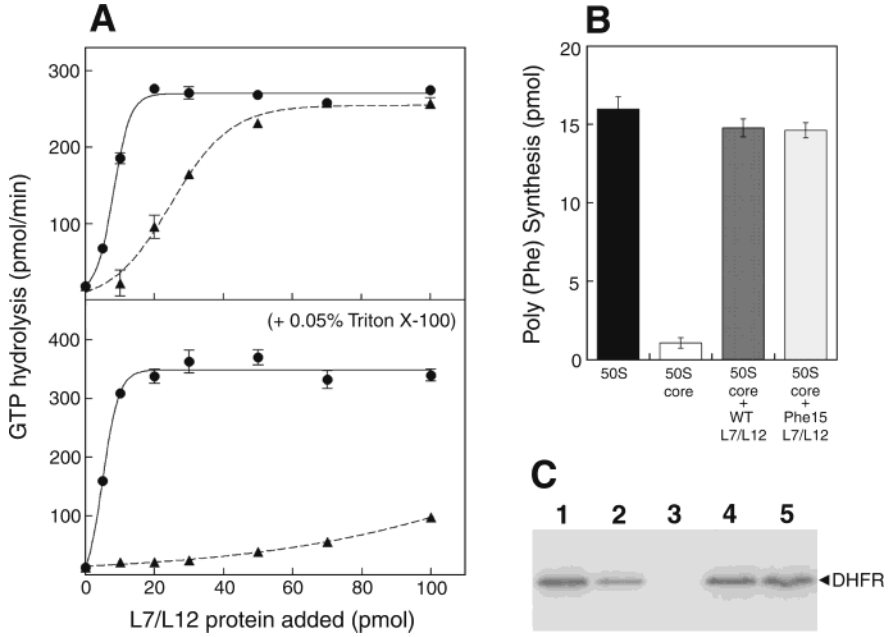

FIGURE 4: Functional analyses of the Phe15-L7/L12. (A) Increasing amounts of wild-type L7/L12 (circles) or Phe15-L7/L12 (triangles) were added to the L7/L12-deficient core particles from Q13 50S subunits in 7 mM $MgCl_2$, 50 mM NH_4Cl, 20 mM Tris-HCl pH 7.6, 5 mM 2-mercaptoethanol in the absence (upper panel) and presence (lower panel) of 0.05% Triton X-100. EF-G-dependent GTP hydrolysis of each sample was tested at 37 °C for 10 min. (B) Wild-type L7/L12 or Phe15-L7/L12 (200 pmol) was added to the core particles deficient in L7/L12 (10 pmol) in 5 mM $MgCl_2$, 120 mM NH_4Cl, 50 mM Tris-HCl pH 7.6 and 0.2 mM DTT, and polyphenylalanine synthesis dependent in EF-Tu/EF-G was tested. (C) Wild-type L7/L12 (lane 4) and Phe15-L7/L12 (lane 5) proteins (400 pmol) were added to L7/L12-deficient core (20 pmol), and the ribosomal activity in translation of natural mRNA for DHFR was assayed using the PURE system, as described in Materials and Methods. The same amounts of wild-type 50S subunits (lane 1), LL103 50S subunits (lane 2), L7/L12-deficient core particles (lane 3) were also tested. lanes 1 and 2, the 50S subunit from Q13 (wild-type) and LL103 strain, respectively; lane 3, L7/L12-deficient core; lanes 4 and 5, the 50S subunit reconstituted from L7/L12-deficient core by adding wild-type and Phe-15 L7/L12, respectively.

clearly the effect of the mutation, the gel analysis was performed in the presence of 0.05% Triton X-100 (Figure 3B), which gave no adverse effect on the functions of ribosomes containing wild-type L7/L12 and ribosomes, as described below (Figure 4A). Unexpectedly, Phe15-L7/L12 showed very low mobility in the gel containing 0.05% Triton X-100 (lane 3), in contrast with normal high mobility of wild-type L7/L12 (lane 2). By mixing L10 with wild-type L7/L12, the L10-L7/L12 complex was formed even in the presence of Triton X-100 (lane 4). No complex formation with Phe15-L7/L12 was detected (lane 5). The mobility of both L7/L12 samples in gel containing Triton X-100 was compared with their cross-linked dimers, which were formed with glutaraldehyde (Figure 3C). Wild-type L7/L12 (left, lane 1) migrated as fast as the sample treated with glutaraldehyde (left, lane 2) that contained the cross-linked dimer (right, lane 2), suggesting that wild-type L7/L12 migrates in the gel as a dimer. The mobility of Phe15-L7/L12 (left, lane 3) was lower than its cross-linked dimer (lelt, lane 4 and right, lane 5) and consistent with that of the protein not cross-linked with glutaraldehyde (right, lane 4). The latter non-cross-linked component may correspond to monomers dissociated during running in the gel, but it is also possible that it may migrate as a dimer whose structural feature is slightly different from the cross-linked dimer. Although this might be clarified by comparison in gel mobility between Phe15-L7/L12 and the authentic L7/L12 monomer, we failed to prepare the monomer by using the Gudkov's method (*21*). Further analysis remains to clarify the detailed state of the mutant L7/L12 in Triton X-100. We inferred that the structure of the Phe15-L7/L12 mutant, and also of its cross-linked dimer, is so extended that it is highly accessible to Triton X-100.

We tested the restoration of ribosome function by adding increasing amounts of L7/L12 samples to 50S core particles deficient in L7/L12. In the absence of Triton X-100, EF-G-dependent GTPase activity was obtained by addition of Phe15-L7/L12 up to a level of activity that was obtained by addition of wild-type L7/L12 (Figure 4A, upper panel). A 3-fold larger amount of Phe15-L7/L12 was required compared to wild-type. In the presence of 0.05 % Triton X-100, only a low level of activity was obtained by addition of 10-fold larger amount of Phe15-L7/L12 compared to wild-type L7/L12 (Figure 4A, lower panel). This result is consistent with the failure of Phe15-L7/L12 to bind to L10 in 0.05% Triton X-100 (Figure 3B). The recovery of ribosome function by Phe15-L7/L12 in the absence of Triton X-100 was also tested in poly(U)-directed polyphenylalanine synthesis. By addition of large excess amounts of Phe15-L7/L12 as well as the wild-type to the 50S cores, the activity was restored to that of wild-type ribosomes (Figure 4B). Similar results were obtained in translation of natural mRNA for dihydrofolate reductase (DHFR) using all isolated translation factors (termed the "PURE system") (*53*). The ribosomal activity was estimated by counting the ^{35}S-labeled products of DHFR. The activity of ribosomes from LL103 cells was about 20−30% (lane 2) of that of wild-type ribosomes (lane 1). Addition of large amounts of Phe15-L7/L12 (lane 5) as well as wild-type L7/L12 (lane 4) to the 50S cores (lane 3) increased the translation activity up to a level comparable to wild-type ribosomes. The results suggest that Phe15-L7/L12, which binds to the 50S subunit in the presence of its

excess amount, exerts normal factor-dependent functions on the ribosome.

DISCUSSION

The present study has focused on the L7/L12 mutant (Ser15 to Phe) that was isolated among spontaneous revertants from streptomycin dependence. Using this mutant, we have demonstrated that Ser15, which is located between helices αl and α2 in the NTD of *E. coli* L7/L12, participates in the formation of a compact dimer structure and binding to L10. The results are in agreement with the crystal structure of isolated *T. maritima* L7/L12 dimer in mode I (*43*), in which the amino acid residue at position 15 (*E. coli* numbering) of one monomer contacts the backbone of position 96 in the CTD of the partner molecule (see Figure 2). It is likely that the Ser to Phe mutation disturbs the NTD−CTD interaction and therefore destabilizes the L7/L12 dimer. The possible contribution of the NTD−CTD interaction, as well as the NTD−NTD binding, to stabilization of L7/L12 dimer is supported by a previous finding that the affinity between the truncated NTDs is 10-fold lower than that between full molecules (*35*). We infer that the Ser15 of L7/L12 is involved in the hydrogen bond that contributes to the NTD−CTD interaction and thus to the formation of the compact L7/L12 dimer.

The NTD−CTD interaction between two wild-type L7/L12 monomers influences the relative orientations of the CTDs, and renders the dimer more compact than an elongated conformation. The model of the L7/L12 dimer from the crystal data is in conflict with previous ones in which the CTD apparently moves through the hinge region located in the middle of molecule (*3*, *33*). This flexible nature of L7/L12 has been supported by many lines of experimental evidence (reviewed in ref *3*). It is therefore likely the L7/L12 dimer is present not only in the compact form shown in the crystal data, but also in the flexible form. Considering the NTD−CTD contacts as well as previous evidence for flexibility of L7/L12, we propose a model that provides for alternative states of the dimer in which L7/L12 seems to be present both in compact and elongated forms in an equilibrium state (Figure 5). The compact form is stabilized not only by the four-helix bundle constructed with helices α2 and α3 of each monomer but also the NTD−CTD contacts, as shown in the crystal structure (mode I). The hinge region exposed in the elongated form is involved in the four-helix bundle as a part of helix α3 in the compact form. It seems that the Phe15-L7/L12 variant differs from the wild-type in direction of the equilibrium state. The elongated form may be predominant in Phe15-L7/L12 because of a defect in the NTD−CTD interaction.

It is well established from electron microscopy that L7/L12 comprises the stalk, an extended structure of the large subunit, in which the CTD is placed at the distal end of the stalk. Protein−protein cross-linking between the CTD and ribosomal proteins located at the stalk base imply either a location for one dimer near the stalk base or the mobility of dimers (*3*). Separate locations for the two dimers have been suggested from results of fluorescence energy transfer (*58*). Moreover, immune electron microscopy using monoclonal anitibodies has shown two distinct locations of the CTD, one at the tip of the stalk and the other at the stalk base

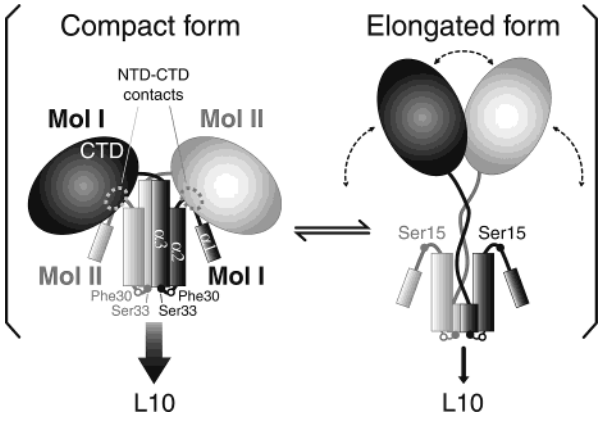

FIGURE 5: Schematic model for changeable states of L7L/12 dimer. There seems to be two L7/L12 dimer states, a highly ordered compact form (left) and an elongated form (right). The compact dimer is stabilized by the NTD−CTD interactions involving Ser15 as well as the four-helix bundle composed of α2 and α3 helix of each monomers. In elongated form, the NTD−CTD contacts disappear, and a part of helix α3 extends and becomes to the hinge region. Compact form seems to bind to L10 with higher affinity than elongated form. Orientation toward the binding protein L10 is taken considering previous finding that *E. coli* Phe30 residue located in a turn between helix α2 and α3 is involved in the interaction with L10 as observed by NMR spectroscopy (*5*), and also that the Ser33 to Cys substitution at this tern loop and its oxidative cross-linking causes a failure in L10 binding (*40*).

(*59*). The latter antibody binding site remains even after one L7/L12 dimer comprising the stalk is released by another antibody (*60*). The stalkless subunits containing one L7/L12 dimer is also prepared by a biochemical approach (*61*). These lines of evidence suggest that there are two locations of the L7/L12 dimer on the ribosome: the CTD of one dimer makes the stalk, and that of the other dimer turns inward to the base region of the stalk (*3*), and that the former dimer binds to the particle with lower affinity than the latter (*58*). It is likely that the compact form of L7/L12 stabilized by the NTD−CTD contacts (Figure 5, left) corresponds to the latter dimer located in the stalk base. The strong binding of this compact form may enhance binding of the former elongated (stalk) form. The present study shows that binding ability of Phe15-L7/L12 to L10 is lower than that of wild-type L7/L12. This can be interpreted in our model by the view that this mutant protein is present predominantly in an elongated state.

The present results also show that the Phe15-L7/L12 although defective in the NTD−CTD interaction can still bind to L10 and function normally in protein synthesis in vitro, even though the binding ability is low. This is consistent with previous findings that L7/L12 dimers cross-linked between CTDs that should not allow the NTD−CTD interaction still bind to the ribosome and show normal function (*40*). An interpretation for these results is that the NTD−CTD interaction is not crucial for the ribosome function, but primarily for assembly onto the subunit. Assembly is still possible by using excess mutant protein. The importance of the NTD−CTD interaction in vivo is suggested by the fact that isolated LL103 ribosomes contain only 25−30% of L7/L12 compared to that of wild-type ribosomes, and showed 15−30% ribosome activity. Our preliminary experiments showed that Phe15-L7/L12 was released more easily than wild-type L7/L12 from the reconstituted ribosomes during subunit isolation by sucrose

density gradient centrifugation (data not shown). This indicates that binding of Phe15-L7/L12 to the ribosomes is weaker and therefore easy to dissociate. It is likely that the affinity of the extended Phe15-L7/L12 for the ribosome is lower than the compact wild-type protein and therefore an excess of Phe15-L7/L12 is required to achieve normal level of ribosome activity. The compact form of L7/L12 dimer may be important not only in its efficient assembly into the GTPase-associated center, but also in retaining the two L7/L12 dimers on the ribosome during the translation cycles.

It is noteworthy that mutant LL103 arose spontaneously as a streptomycin-independent revertant. This mutation may affect the decoding process in cell by reducing L7/L12 amounts (copies) bound to the ribosome. In this regard, the role of L7/L12 in translation accuracy has been reported previously (*56*, *57*, *62*). The present Phe15-L7/L12 variant may be useful in future studies of the functional significance of this multicopy ribosomal protein in vivo.

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