The Accessibility of Yeast Ribosomal Protein L1 as Probed by Proteolysis and Site-directed Mutagenesis Is Different in Intact 60 and 80 S Ribosome

John C. Lee, Cynthia L. Turgeon, and Lee-Chuan C. Yeh

From the Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284

Accessible regions of protein L1 in intact 60 and 80 S ribosomes from Saccharomyces cerevisiae were first detected by controlled proteolysis. The N-terminal region of L1 in either 60 S or 80 S particles, was inaccessible to proteases, but the central and C-terminal regions were accessible. The accessibility of the central region differed depending on the ribosome state. These regions were further examined by determination of the chemical reactivity of specific cysteine residues introduced into these regions by site-directed mutagenesis. All cysteine mutant proteins were capable of binding yeast 5 S rRNA in vitro and the ribosomes containing the mutant proteins were functional in vivo. Residues Cys-257 and Cys-275 were modified in both the 60 and 80 S ribosomes, but the modification rates were different in the two ribosome states. Both residues Cys-62 and Cys-286 were inaccessible in 80 S or 60 S ribosomes. Taken together, the present study identified several accessible regions of L1 in intact ribosomes and further showed that the accessibility of some of the regions was altered upon ribosomal subunit association. The most likely interpretation of these results is that the conformation of the ribosomal protein L1 was altered upon ribosomal subunit association.

Changes in the conformation of the Escherichia coli and eukaryotic ribosome as it binds ligands and participates in protein biosynthesis have been detected. Specific ribosomal proteins and RNA regions that are involved in these changes have also been identified (1-9). For example, base modification studies have identified specific nucleotides in the 5,8, 18, and 28 S rRNA species of mouse Ehrlich ascites cells that are affected upon ribosomal subunit association (10). Tryptic exchange experiments showed that the yeast ribosomes exhibit different global conformation as they participate in protein synthesis (11). However, identification of the ribosomal components that participate in these conformational changes is lacking. Previous studies revealed that the chemical reactivity of specific SH groups on yeast proteins L7 and L26 was altered upon ribosomal subunit interactions, suggesting that the structure and/or environment of these proteins is sensitive to ribosome subunit association (12).

5 S rRNA and its binding protein(s) form a functional domain in the eukaryotic ribosome. Chemical cross-linking studies suggest that the RNA-protein complex is located at the ribosomal subunit interface and participates either directly in the initiation and elongation reactions of protein synthesis or is located in the vicinity of other ribosomal components participating in these reactions (13-19). However, little is known about the structural arrangement of the 5 S RNA-binding protein in the intact ribosome. It is also not known whether the structural organization of the protein or its environment undergoes changes as the eukaryotic ribosome participates in the different stages of protein synthesis.

Controlled proteolytic digestion has been widely used as a probe for studying protein structure-function relationships (see Ref. 20 for review) including prokaryotic ribosome structure (21-25). Results obtained by this approach agree reasonably well with data obtained by other experimental techniques, such as RNA-protein and protein-protein cross-linking (26, 27), immunoelectron microscopy (28, 29), and neutron scattering (30, 31).

The aim of the present study is to examine the structural arrangement of the yeast 5 S RNA-binding protein L1 in the intact 60 and 80 S ribosome. Two experimental approaches were used: (i) controlled proteolytic digestion of intact ribosome, followed by polyacrylamide gel electrophoresis and Western blot analysis with specific anti-HA antibody to analyze the fate of protein L1; (ii) monitoring the chemical reactivity of specific sulfhydryl groups introduced, by site-directed mutagenesis, into the C-terminal region of the L1 molecule.

EXPERIMENTAL PROCEDURES

Materials and Yeast Strains—All chemicals were reagent grade. 5-iodoacetamidofluorescein (IAF)1 was purchased from Molecular Probes, Inc., OR. Chymotrypsin and endoproteinase Glu-C (V8 protease) were purchased from Sigma. (-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington. Phenylmethylsulfonyl fluoride and p-nitrophenyl-p-guanidinobenzoate were purchased from Sigma. The yeast strain LY1191 (MATa ura3-52 trp1-Δ101 lys2-801 leu2-Δ1 his3-Δ200 rpl1-Δ1:TRP1 pRS315-RPL1-HA) was used for the controlled proteolysis studies. LY1191 was derived from JWY3707 in which the genomic copy of RPL1 was disrupted (32). Plasmid pRS315-RPL1-HA contained an epitope-tagged allele of the RPL1 gene with a nucleotide sequence coding for the 9-amino acid hemagglutinin (HA) epitope inserted between codons B and 9 (33).

Preparation and Proteolytic Digestion of 60 and 80 S Ribosomes—Yeast cells (LY1191) were grown in YEPD at 30 °C to mid-log phase and harvested immediately following addition of cycloheximide (5 mg/ml of medium). After washing, cells were broken by vortexing with glass beads in buffer A (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 30 mM MgCl2, 0.2 µM diethyl pyrocarbonate, and 200 µM EDTA). Ribosomes were collected by high speed centrifugation, dissolved in buffer B (50 mM Tris-HCl, pH 7.7, 8 mM MgCl2, 650 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA), pelleted and dissolved in buffer C (50 mM Tris-HCl, pH 7.7, 8 mM MgCl2, 50 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA) to reform 80 S particles. 60 S subunits were purified by gradient centrifugation in high salt buffer B as described (12). Ribosome samples

1 The abbreviations used are IAF, 5-iodoacetamidofluorescein; HA, hemagglutinin.
were analyzed by analytical gradient centrifugation to confirm that they were 80 S or 60 S particles. Ribosomes (8 A260/100 μl of buffer C) were digested with varying concentrations of chymotrypsin, trypsin, or V8 at 25 °C for the duration indicated. Reactions were stopped by the addition of p-nitrophenyl-p-guanidinobenzoate (final concentration 0.1 mM for trypsin) or phenylmethylsulfonyl fluoride (10 mM for chymotrypsin) and analyzed immediately as described below.

Analysis of Digestion Products by Gel Electrophoresis and Western Blot Analysis—Digested ribosomes were mixed with Laemmli sample buffer, boiled for 3 min, and loaded onto SDS-containing polyacrylamide gels. After electrophoresis, the digested peptides were transferred to nitrocellulose membrane (Schleicher & Schuell). The identity of the labeled mutant protein L1 was further confirmed by Western blot analysis using the anti-HA epitope 12CA5 mouse monoclonal antibody (Berkeley Antibody Co.). The molecular weight of the products was determined using a fluorescent, mid-range molecular weight protein standard (Diversified Biotechnology) containing phosphorylase b, bovine serum albumin, alcohol dehydrogenase, carbonyl hydrdride, trypsin inhibitor, and lysozyme (95, 68, 39, 29, 20.4, and 14 kDa, respectively).

Substitution of Amino Acids with Cysteine in L1 by Site-directed Mutagenesis—Specific mutations were introduced into the RPL1 gene cloned in pRS315-RPL1-HA using polymerase chain reaction (32). The following mutagenic primers were used for generating the mutants E257C, T275C, and V268C: 5'-AAGCACAATTGCAAGAATTGCC-3', 5'-CGCAATTGCGCTGCTGCTGCTGCTGCAAG-3', and 5'-GCTGCCAAG-3', respectively. In each case, the sequence of the entire RPL1 gene was determined to ensure that it contained only the desired mutation. The resultant mutant plasmid was shuffled into yeast strain J WY3707 and selected first on C-Ura-Leu+Gal plates and then on S-fluoroorotic acid-containing plates. The yeast strains harboring the mutations E257C, T275C, and V268C in protein L1 were named LY257C, LY275C, and LY268C, respectively. The genotypes were MATα ura3-52 trpl-1A01 lys2-801 leu2-11 his3-1200 rpl1-1::TRP1 + pRS315-rpl1-HA-257C, MATα ura3-52 trpl-1A01 lys2-801 leu2-11 his3-1200 rpl1-1::TRP1 + pRS315-rpl1-HA-275C, and MATα ura3-52 trpl-1A01 lys2-801 leu2-11 his3-1200 rpl1-1::TRP1 + pRS315-rpl1-HA-286C, respectively.

Chemical Modification of Intact 60 S Subunits and 80 S Ribosomes with IAF—Ribosomes (100 A260/ml) were labeled with 1 mM IAF at 0 °C for 2 h in the dark as described previously (12) with the following modifications. The labeling reaction was stopped by the addition of cysteine to a final concentration of 10 mM. The labeled ribosomes were purified by chromatography on a Sephadex G-25 column. Fractions containing the labeled ribosomes were pooled and subjected to centrifugation at 55,000 rpm for 2 h at 4 °C in a 75Ti rotor through a sucrose gradient. Ribosomes (80 S or 60 S particles) was followed by SDS-polyacrylamide gel electrophoresis under controlled conditions as described below.

Analysis of CNBr Fragments—Total 80 S ribosomal proteins were resolved on SDS-polyacrylamide gels. The IAF-labeled L1 was visualized under the UV light after electrophoresis, excised from the gel, and eluted with 50 mM ammonium bicarbonate, pH 7.8, containing 0.1% SDS. They were concentrated in a Microcon-10 Ultrafree-MC microconcentrator (Amicon, Inc.). The protein was recovered by acetone precipitation and dissolved in 70% formic acid containing 10 mg/ml freshly prepared CNBr. After incubation for 18 h at room temperature, the reaction mixture was dried under a stream of nitrogen. The dried digest was dissolved in Laemmli sample buffer and analyzed on SDS-polyacrylamide gels as described above. The pattern of CNBr fragments was first viewed under UV lights and then stained with Coomassie Brilliant Blue R250.

RESULTS
Saccharomyces cerevisiae ribosomes containing the HA-tagged ribosomal protein L1 were digested with three different proteolytic enzymes under controlled conditions as described under “Experimental Procedures.” The fate of protein L1 in either 80 S or 60 S ribosomes was followed by SDS-polyacrylamide gel electrophoresis and immunoblotting using a specific monoclonal anti-HA antibody. Since the tagged epitope (HA) is inserted between amino acids 8 and 9 of protein L1 and the antibody in use is directed toward the HA epitope, only those peptide fragments containing the N-terminal region of L1 would be detected by the present approach. Control experiments in which the enzyme was incubated with the enzyme inhibitor and boiled prior to addition of ribosomes showed that the arresting condition used was sufficient to stop the protease.

Fig. 1A shows a typical Western analysis of chymotryptic digests of the 60 S subunits. Three immunoreactive protein L1 fragments (F1, F2, and F3 with estimated molecular masses of 20, 16.5, and 15 kDa, respectively) were observed. The reaction conditions were chosen so that the digestion was incomplete in order to observe primary cleavages instead of secondary cleavages. L1 was completely digested by higher concentrations of enzymes (data not shown). Occasionally, several minor, immunoreactive species, such as those migrating slightly faster than the parent L1, were detected on these membranes. These species were present in the time zero undigested samples and were not considered further. Fig. 2 shows that the digestion patterns followed a time-dependent progression of cleavage. A possible cleavage pathway appeared to be as follows: 34 kDa (L1) → 20 kDa → 16.5 kDa → 15 kDa.

To assess the effect of 40 S binding on these accessible regions of protein L1 in the 60 S subunit, 80 S ribosomes were digested by chymotrypsin. As shown in Fig. 1B, no immunoreactive fragments of L1 were detectable in the digest of the 80 S ribosome. Similar results were observed when 80 S ribosomes were digested at a higher concentration of chymotrypsin. Hence, the primary chymotryptic cleavage site (cleavage at which resulted in the 20-kDa fragment) on protein L1 in the 60 S subunit became inaccessible upon binding with the 40 S subunit.

Whereas trypsin digest of the 60 S subunits produced two immunoreactive L1 fragments (F1 and F2), digestion of 80 S ribosomes produced three fragments (F1, F2, and F3) (Fig. 3, A and B). The estimated molecular masses of F1 and F2 from the 60 S digest are 32 and 30 kDa, respectively, F1, F2, and F3 from the 80 S digest are 32, 31, and 30 kDa, respectively. Although there were numerous potential trypsin cleavage sites.
scattered throughout the protein L1 molecule, only a few were cleaved by trypsin under the current experimental conditions. These sites are located near the C-terminal region of L1. Higher concentrations (up to 3-fold) of trypsins did not result in new cleavages. Fig. 4 shows the kinetics of appearance of the tryptic fragments. It appeared that both the 32-kDa and the 30-kDa fragments from the 60S subunits were generated about the same time. Both cleavage sites in L1 in the 60S subunit were almost equally accessible to trypsin. On the other hand, digestion of protein L1 in the 80S ribosomes showed a different time-dependent progression, i.e., 34 kDa (L1) \(\rightarrow\) 32 kDa \(\rightarrow\) 31 kDa \(\rightarrow\) 30 kDa. Thus, the 31-kDa fragment was a unique digestion product of protein L1 in the 80S ribosome.

Fig. 5 shows a typical Western analysis of peptides from V8 protease digestion of 60S (A) or 80S (B) ribosomes. Conditions were similar to those described in the legend of Fig. 1. The estimated molecular mass values for F1 and F2 in panel A are 32 and 30 kDa, respectively. Those for F1–F3 in panel B are 32, 31, and 30 kDa, respectively. Several nonspecific bands appeared in the undigested control as well as in the experimental samples. These bands were not considered further. Although there were numerous potentially susceptible cleavage sites in protein L1, only one was accessible in either 60S (Fig. 5A) or 80S (Fig. 5B) particles under the present experimental conditions. Unlike the results of tryptic and chymotryptic digestions, which showed that the cleavages occurred near the C terminus, V8 digestion of the ribosomes resulted in a single cleavage near the middle of the protein producing a 17-kDa fragment. The intensity of the 17-kDa fragment increased with time of digestion. However, a comparison of the kinetics of appearance of the 17-kDa fragment revealed that the particular cleavage of L1 occurred more readily in the 80S particle than in the 60S subunit (Fig. 6). The observation suggested that the environment or the structure at or near this cleavage site in protein L1 had undergone a significant change, making it more susceptible to V8 in the 80S than in the 60S subunits.

The accessibility of the different L1 regions in the intact ribosome was also probed by measuring the chemical reactivity of specific cysteine residues in L1. The only cysteine at residue 62 in the wild-type protein L1 was not available to react with IAF in either the 60S subunit or the 80S ribosome (Fig. 7A,
mean of 2 and Lanes 1 conditions at 2 or 4 mM MgCl2 (data not shown). As shown modifications when the 60 S subunit was subjected to unfolding lanres 1 protein L1 from 60 S (\[\text{molecular weights indicated on the right side of the figure.}\]

Each point represents the mean of \(n = 4\).

**Fig. 6.** Kinetics of appearance of the V8 peptide fragments of protein L1 from 60 S (A) and 80 S (B). Each point represents the mean of \(n = 4\).

**Fig. 7.** IAF labeling of 60 and 80 S ribosomes. A, IAF-labeled total ribosomal proteins were analyzed on SDS-containing polyacrylamide gels, transferred onto membranes, and viewed under a UV light. B, Positions of the labeled proteins were marked on the membrane with a needle. The blue bands were the residual mutant L1; \(\text{lane 1}\), wild-type yeast strain; \(\text{lane 2}\), mutant L1 with T275C substitution; \(\text{lane 3}\), fluorescent molecular weight standards. \(\bullet\) denotes the residual mutant L1; \(\bullet \bullet \) denotes the fluorescent 10-kDa CNBr-digested fragment.

Mutation of L1 proteins (E257C, T275C, or V286C) with a single cysteine substitution at position 257, 275, or 286, respectively, were produced. These mutant proteins were capable to bind 5 S rRNA as well as the wild-type (data not shown) in the in vitro system. The mutant proteins were incorporated into the 60 S subunits as shown by Western analysis of total ribosomal proteins. Moreover, yeast strains (LY257C, LY275C, and LY286C) expressing these mutant proteins were viable. Their growth rates were indistinguishable from that of the wild-type (data not shown). These in vitro and in vivo data strongly suggest that ribosomes containing these mutant protein L1 were either unaltered or only minimally disrupted structurally.

Purified 80 S or 60 S ribosomes were obtained from each mutant yeast strain and subjected to IAF labeling. Fig. 7A shows the fluorescence patterns of the IAF-tagged proteins from 60 S or 80 S ribosomes as analyzed on SDS-polyacrylamide gels. In addition to L7 and L26, which were labeled in the wild-type 80 S ribosomes, a protein with a molecular mass of 34 kDa was labeled with IAF in ribosomes from the yeast strains LY257C (lanes 3 and 4) and LY275C (lanes 5 and 6). That the 34-kDa protein in both mutant proteins was indeed protein L1 was confirmed by Western blot analysis (Fig. 7B).

Residue Cys-257 was labeled in the 60 and 80 S ribosomes. However, kinetic labeling studies showed that the residue in the 60 S subunit reacted with IAF slightly faster than that in the 80 S ribosome. Residue Cys-257 in the mutant protein L1 reacted with IAF in both the 60 and 80 S particles, and it reacted more rapidly in the 80 S ribosome than in the 60 S particle. These observations suggested that residue Cys-257 might be more exposed in the 60 S subunit than in the 80 S ribosome and that residue Cys-275 became more exposed in the 80 S than in the 60 S. Residue Cys-286 in protein L1 in either the 60 S or the 80 S particle could not be labeled with IAF.

To check that the single cysteine substitution at residue 257 or 275 had not affected the reactivity of Cys-62, the IAF-labeled mutant proteins (E257C or T275C) were isolated from the SDS-containing polyacrylamide gels and subjected to CNBr deavages. Two peptide fragments (24 and 10 kDa) were produced in both mutant proteins. Only the 10-kDa fragment was fluorescently labeled (Fig. 8, lanes 1 and 2). Since protein L1 contains only one methionine residue at position 208, CNBr treatment of L1 should produce a 24-kDa fragment containing the N-terminal region of L1 and a 10-kDa fragment containing the C-terminal region. The observation thus implied that Cys-62 was not labeled and the labeled residue in the mutant protein E257C or T275C was 257 or 275, respectively.
DISCUSSION

In the present study, the accessible regions of the yeast ribosomal protein L1 in intact ribosomes were probed. The combination of controlled proteolysis and subsequent immunoblotting facilitated examination of the topography and topographical changes of a specific tagged protein among the many proteins and RNA species in the ribosome complex.

Protein L1 in intact 60 S ribosomal subunits was relatively resistant to proteolysis with only a few accessible regions. The N terminus of protein L1 in the 60 S subunit was not cleaved, but the middle and the C-terminal region of the protein were available for cleavages. Chemical modifications also suggested that the N-terminal region of L1, and particularly the Cys-62 residue, in both the 60 and 80 S particles was not exposed. Results of the unfolding experiments by exposing the ribosome to lower magnesium concentrations also suggested that the N terminus is buried in the ribosome. The data agreed with an earlier study that the N-terminal L1 region in the 60 S subunit or the 80 S ribosome was not accessible to a monoclonal antibody (33). By comparison, in the isolated 5 S rRNA-L1 protein (RNP) complex, the first 20 amino acids of L1 and the C-terminal region were accessible to dithymotrypsin and trypsin, respectively (35).

In E. coli ribosomes, three proteins (EcoL5, EcoL18, and EcoL25) bind 5 S rRNA. The 5 S rRNA-protein complex lies in the central protuberance of the 50 S ribosomal subunit. Proteolytic digestion of the E. coli ribosomes with endoproteinases Lys-C, Glu-C, chymotrypsin, and trypsin showed that Ecol18 and EcoL25 were protected but EcoL5 was accessible to proteases (22). The N-terminal region of Ecol18 in the intact E. coli ribosome was not accessible to protease, but in the isolated complex the region was readily accessible to trypsin (36). The N-terminal regions of the Ecol18 and yeast protein L1 contain an unusually high number of basic amino acids and show some degree of homology (37, 38). Although the exact function of the N-terminal region of the 5 S rRNA-binding protein is unknown at the present, studies on the E. coli ribosome suggest that the N-terminal region of Ecol18 may be involved in an interaction with the 23 S rRNA molecule holding the 5 S rRNA-protein complex to the ribosome (36, 37). Experiments are in progress to determine the function of the N terminus of the yeast ribosomal protein L1.

In E. coli, there appears to be a positive correlation between the order of assembly of ribosomal proteins and their susceptibility to proteolysis and their ease of removal with high concentration of salt. Most proteins that are essential for in vitro early assembly are inaccessible to proteolysis (22). Conversely, proteins that have extended surface domains and are accessible to proteases are late assembly proteins. A similar correlation has been suggested for the yeast ribosome (39). Yeast protein L1 can be removed from intact ribosomes with a relatively low concentration of ammonium chloride (40). These data collectively would suggest that protein L1 might be a late assembly protein. Published data indicate that in mammalian cells and Xenopus oocytes, L5, the homolog of yeast L1, forms a stable complex with 5 S rRNA prior to assembly into ribosomal subunits (41–43). Were binding of yeast ribosomal protein L1 to 5 S rRNA also a prerequisite for assembly into 60 S subunits, the present results would suggest that the RNA-protein complex is assembled into the 60 S subunit at a late stage of ribosome biogenesis.

Binding of 40 S subunits to the 60 S subunits has been shown to result in a conformational change in the yeast ribosome (11, 12). The present study revealed that ribosomal subunit association affected the accessibility of several regions in protein L1. Notably, the middle of the protein molecule became accessible upon dissociation of the 80 S ribosome to its 60 and 40 S subunits. One possible explanation of the observation is that this region of L1 was involved directly in the interaction with the 40 S subunits and was protected from proteolysis by the physical presence of the 40 S subunit. Alternatively, the region had undergone a structural alteration resulting in a shielding of the region from proteolysis. The current results could not distinguish between these two possibilities at present. That the Cys-257 was modified slower in the 80 S than in the 60 S subunit would suggest that the structure of L1 at or surrounding Cys-257 residue was tightened in the 80 S ribosome compared to that in the 60 S. Our data also suggest that the Cys-257-containing region appeared to be more hindered in the 60 S than in the 80 S ribosome. Furthermore, the higher relative fluorescence intensity of the IAF-labeled L1 with Cys-257 compared to that of L1 with Cys-275 implied that the former was more exposed and the latter was only partially exposed.

The amino acid replacement studies indicated that residues Glu-257, Thr-275, or Val-286 could be replaced by a cysteine residue without affecting protein function to any detectable extent. The result of the E257C mutant was somewhat surprising in view of the nature of the substitution (replacing a negatively charged side chain with a neutral one). On the other hand, a proline residue is found at this position in all the other known eukaryotic 5 S rRNA-binding proteins. Theoretical predictions of the secondary structure of protein L1 indicate that the residue has a very high probability to be located in a loop region. The other two substitutions were of a more conservative nature and were not expected to cause a major structural disruption. Residue Thr-275 is not conserved, whereas Val-286 is highly conserved among the known eukaryotic 5 S rRNA-binding proteins.

In conclusion, our results indicated that the N terminus of protein L1 was not accessible in both the 60 S subunit and the 80 S ribosome. The C-terminal region, a region that is partly involved in 5 S rRNA binding, was readily accessible. A central segment of L1 appeared to be dynamic and was readily accessible in the 60 S subunit but became inaccessible in the 80 S. The observation is in agreement with the notion that the ribosome is a highly flexible structure with dynamic properties that are prerequisites for function. The described method of combined proteolysis and site-directed mutagenesis and specific labeling has been a powerful tool for probing the structural arrangement of protein L1 in intact ribosomes. The approach has been also useful in monitoring changes in specific regions of L1 as the ribosome participates in the different stages of protein synthesis. This experimental approach may also be useful in mapping structures of a protein in other complex structures.

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