Cryo-Electron Microscopic Localization of Protein L7/L12 within the Escherichia coli 70S Ribosome by Difference Mapping and Nanogold Labeling

Luisa Montesano-Roditis¹, Dohn G. Glitz¹, Robert R. Traut², and Phoebe L. Stewart³*

¹Department of Biological Chemistry, UCLA School of Medicine, Box 951737, University of California, Los Angeles, CA 90095-1737, USA

²Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 96517, USA

³Department of Molecular and Medical Pharmacology, Crump Institute for Molecular Imaging, UCLA School of Medicine, A-324 CIMI, Box 951770, University of California, Los Angeles, CA 90095-1770, USA.

*Corresponding author:
Dr. Phoebe L. Stewart
Phone: 310-206-7055
Fax: 310-206-8975
Email: pstewart@mednet.ucla.edu

Running Title: Cryo-EM Localization of a Ribosomal Protein
Summary

The *Escherichia coli* ribosomal protein L7/L12 is central to the translocation step of translation and it is known to be flexible under some conditions. Assignment of electron density to L7/L12 was not possible in the recent 2.4 Å resolution x-ray crystallographic structure (Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000) *Science* **289**, 905-920).

We have localized the two dimers of L7/L12 within the structure of the 70S ribosome using two reconstitution approaches together with cryo-electron microscopy and single particle reconstruction. First, structures were determined for ribosomal cores, from which protein L7/L12 had been removed by treatment with NH₄Cl and ethanol, and for reconstituted ribosomes, in which purified L7/L12 had been restored to core particles. Difference mapping revealed that the reconstituted ribosomes had additional density within the L7/L12 shoulder next to protein L11. Second, ribosomes were reconstituted using an L7/L12 variant in which a single cysteine at position 89 in the C-terminal domain was modified with Nanogold™, a 14 Å gold derivative. Reconstruction from cryo-EM images and difference mapping placed the gold at four interfacial positions. The finding of multiple sites for the C-terminal domain of L7/L12 suggests that the conformation of this protein may change during the steps of elongation and translocation.

Key words: ribosome; cryo-electron microscopy; image reconstruction; protein L7/L12; Nanogold; protein localization.
Introduction

The ribosome is the platform for all protein synthesis and the catalyst of peptide bond formation. The centrality of the ribosome is shown by its universality, its conservation throughout all forms of life, and its frequent targeting by toxins and antibiotics (1). It has been the subject of scores of studies that have linked the structure of the particle with its mechanism of action in protein synthesis. Traditional electron microscopy studies have played a central role in allowing visualization of the ribosome and its subunits, and in the placement of component proteins, segments of its large RNA molecules, and functional sites (2,3).

Cryo-electron microscopy adds new dimensions to these investigations while confirming the basic observations of the earlier work; it allows description of a native particle, frozen in vitreous ice and undistorted by drying and staining, and it permits visualization of the interior of the particle rather than simply its stained or shadowed surfaces. Cryo-EM reconstructions of the ribosome have been generated through independent investigations in two different laboratories. This work has resulted in the attainment of greater resolution in the description of the overall structure of the ribosome (4,5), and also has allowed structural studies of ribosomes complexed with tRNAs and protein factors (6-8). More recently, cryo-EM data have aided in the determination of phases for crystal structures of the 30S subunit (9), the 50S subunit (10) and the 70S ribosome (11). Crystal structures of the 30S (12,13) and 50S (14) subunits have been solved at 3.0-3.3 and 2.4 Å respectively, and a 7.8 Å structure of a 70S functional complex has been reported (11).

Electron density for protein L7/L12 was not apparent even in the recent 2.4 Å resolution 50S ribosome structure (14) and the molecule’s exact position within the ribosome remains nebulous. The protein L7/L12 is central to the translocation step of translation and it is the only ribosomal protein that is present in multiple copies [reviewed in (15)]. The monomer of L7/L12 has a molecular mass of 12 kDa and is organized in the ribosome as two dimers bound to one copy of protein L10, which in turn anchors the
pentamer to the large subunit. The L7/L12 monomer includes two distinct structural
domains thought to be linked by a flexible hinge (residues 37-52); the elongated, helical
N-terminal domains (residues 1-36) are responsible for dimer interaction and binding to L10,
and the larger, globular C-terminal domains (residues 53-120) interact with elongation
factors. The crystal structure of the E. coli L7/L12 C-terminal domain has been solved at 1.7
Å resolution (16). Models of the E. coli L7/L12 indicate that the dimer can span up to 125
Å when extended (17). A recent crystal structure of L12 from the hyperthermophilic
bacterium T. maritima shows two alternative conformations for the hinge region: an
extended coil or a long α-helix that folds back on the N-terminal domain forming a compact
overall protein structure (18). The flexible hinge conformation is in better agreement with
the body of evidence that indicates the E. coli L7/L12 protein has a great deal of overall
flexibility (19).

L7/L12 is easily and selectively dissociated from and reconstituted into the ribosome
(20). When it is removed one of the most recognizable features seen in images of
negatively stained 50S particles, the stalk (or arm), disappears. The stalk and full activity
are restored when L7/L12 is added back, and ribosome nomenclature has equated L7/L12
and the stalk. A body of evidence suggests that L7/L12 exists in at least two
conformations and its mobility is essential for its function (19,21,22).

The experiments described in this paper demonstrate a direct approach to the
placement of ribosomal proteins that is based on ribosome reconstitution and is related to
the classic “single omission reconstitutions” of Nomura (23). The cryo-EM structure of 70S
ribosomes that lack a single protein component, L7/L12, is compared to that of
reconstitutes that include the protein. Some conformational changes are observed in the
ribosome upon binding of L7/L12 in regions previously noted to be conformationally
flexible (e.g. (4,5). In addition, we have incorporated a variant form of L7/L12 that has
been modified with Nanogold™, a 14 Å gold complex (24) that is easily seen in cryo-
electron micrographs. Difference mapping, together with Nanogold labeling, places the N-
terminal domains of protein L7/L12 on the 50S subunit body next to protein L11; and indicates four allowed sites for the L7/L12 C-terminal domains.
Experimental Procedures

Preparation and Characterization of Ribosomes

E. coli ribosomes were isolated (25) and tight couples prepared (26) as described. Treatment of 70S ribosomes with 0.5 M NH₄Cl and 50% ethanol at 0°C dissociated protein L7/L12 and generated cores that were isolated by sedimentation (27). Recombinant wild type and Cys-89 L7/L12 were prepared as previously described (28). Incubation of 70S core particles with an 8-fold molar excess of either native or Nanogold-modified L7/L12 yielded wild type and Nanogold-modified reconstituted ribosomes respectively. Wild type reconstitutes were separated from free protein by sedimentation through a 10% sucrose pad (28). Nanogold-modified reconstituted 70S were isolated on 10-30% sucrose gradients in 10 mM Tris-HCl, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂ by centrifugation in a Sorvall AH-650 rotor at 40,000 rpm for 3 h at 4°C; fractions which contained 70S ribosomes were used for microscopy after dialysis against 10 mM Tris-HCl, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂. Ribosomes and reconstituted ribosome preparations were analyzed by electron microscopy of negatively stained samples as described (25). To observe 50S subunits, portions of our ribosome preparations were dissociated by incubation in buffer containing 1 mM Mg²⁺ and negatively stained. Methods used in the extraction of ribosomal proteins (29) and their analysis by reverse-phase HPLC (30) and mass spectrometry (31) were adapted from the published procedures.

Preparation of Nanogold-Labeled Protein L7/L12

Immediately after reduction with 1% 2-mercaptoethanol in 10 mM Tris-HCl, pH 7.5, 200 mM NH₄Cl for 1 h at 37°C and removal of the reducing agent by passage through two successive Bio-Rad Bio-Spin 6 columns equilibrated in 20 mM sodium phosphate, pH 6.5, approximately 4 nmoles of recombinant Cys-89 L7/L12 in 70-80 µl of 20 mM sodium phosphate, pH 6.5 were added to an excess of monomaleimido-Nanogold reagent
Nanoprobes Inc., Stony Brook, NY) suspended in 30 µl of isopropanol. Water was added to obtain a final volume of 180 µl and the reaction mixture was incubated for 1 h at room temperature. Nanogold-modified protein was isolated by size exclusion HPLC on a Beckman Spherogel-TSK 2000 SW column (7.5 x 300 mm) at a flow rate of 1 ml/min. 20 µl aliquots of each of the 500 µl fractions were run on duplicate 12.5% Laemmli SDS-PAGE gels (32). One gel was stained for proteins with silver stain (Bio-Rad), the other for Nanogold with the LI Silver stain (Nanoprobes Inc., Stony Brook, NY). The fractions with the highest concentration of separated labeled protein were concentrated and used for ribosome reconstitution as described above.

Cryo-electron microscopy

Ribosome samples were prepared for cryo-electron microscopy according to published procedures (33,34). A 5 µl droplet of sample was applied to a holey carbon coated molybdenum grid that was freshly coated with a thin (~ 20 Å) carbon film; after 30-50 seconds, it was blotted from both sides for 2-3 seconds (35), and plunged into an ethane slush. The grid was immediately transferred to and stored in liquid nitrogen. Microscopy was performed on a Philips CM120 transmission cryo-electron microscope (FEI, Hillsboro, OR) equipped with a LaB₆ filament, a Gatan (Pleasanton, CA) cryo-holder, and a Gatan slow-scan CCD camera (YAG scintillator, 1024 x 1024 pixels). Images were collected under low dose conditions (<20 electrons/Å²) at a nominal magnification of 45,000x and at three defocus levels (-0.5 µm, -1.0 µm, -1.5 µm). The digital images have a pixel size of 4.1 Å, as determined by calibration with a catalase crystal.

Image Processing

Interactive selection of individual ribosome images as 100x100 pixel fields, exclusion of density from nearby particles, and the application of a circular mask were performed with the QVIEW software package (36). Subsequent processing was
performed with the IMAGIC software package (37). Initially a set of 4447 images of 70S
tight couple particles collected at -1.5 µm defocus, low-pass filtered to 30 Å and density
inverted, were translationally aligned, then subjected to multivariate statistical analysis and
classification essentially as described in (38) to obtain 500 class-sum images. The Euler
angles of the 347 class-sum images with the highest membership were calculated by
angular reconstitution and a preliminary three-dimensional reconstruction was calculated by
exact filtered back-projection (39). After three rounds of refinement by the anchor set
method (37), the three-dimensional reconstruction of the 347 class-sum images was used
as an initial reference to find the Euler angles of the individual 4447 particle images, low-
pass filtered to 23 Å. After two rounds of refinement, the final reconstruction of these
images was used in turn as an initial reference model for the determination of the Euler
angles of images of L7/L12 reconstituted ribosomes and core particles.

Images collected at three different defocus values (-0.5 µm, -1.0 µm, -1.5 µm) were
combined analytically after deconvolution and correction for the CTF of the electron
microscope as described (38), except the decay constant was 25 nm², and the Fermi filter
resolution cutoff was 1/8.2 Å⁻¹. The L7/L12 reconstituted ribosomes and core particles data
sets each underwent three rounds of anchor set refinement. The final reconstruction of
L7/L12 reconstituted ribosomes included data from 8565 images, and that of the core
particles included data from 9739 images. Euler angles for 486 CTF-corrected particle
images of Nanogold-labeled ribosomes were determined by using the wild type L7/L12
reconstituted ribosome reconstruction as a reference. After calculating a preliminary
Nanogold-labeled reconstruction, a subset of the best 317 Nanogold-labeled particle
images was selected by comparison of the particle images with their corresponding
reprojections.

The resolution of the reconstructions was calculated by the Fourier shell correlation
method, splitting the data sets into two halves, calculating two half-reconstructions, and
assuming the 0.5 correlation threshold as the resolution limit (40). Ribosome reconstructions
were displayed with the AVS visualization package (Advanced Visualization System, Inc.) at isosurface threshold levels chosen to include a volume of $2.9 \times 10^6 \, \text{Å}^3$. The difference map between the wild type L7/L12 reconstituted ribosome and the core ribosome was calculated in IMAGIC after filtering the input maps to 26 Å resolution. Difference maps were also calculated using reconstructions obtained from randomly generated halfsets of our core and reconstituted ribosome images. These maps gave the same results as the one with the entire data sets. Difference density not clearly associated with the ribosome was removed with a circular mask (260 Å diameter) and small regions of disconnected density were computationally removed. The difference density between the reconstituted and core ribosome reconstructions is displayed with the isocontour level set at 3 standard deviations ($3\sigma$) above the mean value.

The resolution of the Nanogold-labeled reconstruction was evaluated both by the FSC between two half-reconstructions and by the FSC between the full, non-split Nanogold reconstruction with the full reconstruction of the wild type L7/L12 reconstituted ribosome. Difference maps were calculated between the Nanogold-labeled ribosome and the wild type reconstituted ribosome after filtering the input maps to either 45 Å or 60 Å resolution. The 45 Å resolution Nanogold difference density is displayed with the isocontour level set at 5 standard deviations ($5\sigma$) above the mean value.
Results

Preparation and characterization of cores and reconstituted ribosomes

E. coli ribosomes and tight couples were prepared by well established procedures (25). L7/L12 is easily extracted from ribosomes as detailed in (20,22); we chose the gentlest possible condition that afforded maximal removal of L7/L12 and minimal extraction of other ribosomal proteins. 70S tight couples were treated with NH₄Cl and ethanol on ice and the resulting 70S ribosome cores were isolated by sedimentation. It is well established that L7/L12 forms the characteristic stalk observed in negatively stained 50S subunits (41). To confirm the removal of L7/L12 in our core preparation, we used EM to examine negatively stained 50S subunits of our ribosome preparations. In the tight couple 50S subunits the L7/L12 stalk was readily identified, while in the core 50S subunits a complete absence of stalks was noted. To establish that recombinant wild type L7/L12 or Nanogold-labeled L7/L12 is incorporated specifically into ribosomes, we viewed 50S subunits of the reconstitutes and observed nearly complete restoration of stalks.

The protein composition of our ribosome preparations was confirmed by HPLC and electrospray-ionization mass spectrometry. Ribosomal proteins were extracted from 70S tight couples, 70S cores, and L7/L12 70S reconstituted ribosomes and analyzed by reverse-phase HPLC. In Figure 1A, the arrow identifies a double peak that elutes last in the chromatogram of the tight couple ribosomal protein extract. This double peak elutes at the same position as a purified L7/L12 standard and contains species of molecular mass compatible with protein L7/L12, as determined by mass spectrometry. The elution profile of an extract from 70S cores lacks the L7/L12 double peak (Figure 1B), while the L7/L12 double peak is restored in the chromatogram of the protein extract from L7/L12 reconstituted ribosomes shown in Figure 1C. No other major differences were observed among the chromatograms. Also, analysis of the supernatant of the NH₄Cl / ethanol extract of tight couples revealed that protein L7/L12 accounted for about 95% of the protein; of the
minor peaks, none was compatible with ribosomal proteins. We conclude that our means of extraction of L7/L12 yields cores that are greatly depleted in protein L7/L12 while not removing any significant amounts of other ribosomal proteins. In addition, the cores incorporate purified wild type or Nanogold-labeled L7/L12 protein efficiently. The level of L7/L12 in the reconstituted ribosomes was estimated to be ~75% by evaluating the relative peak areas in the HPLC profiles and >90% by EM of negatively stained samples.

Structures of wild type L7/L12 reconstituted ribosomes and of 70S core particles, and difference mapping

Our goal was to separately determine and then compare the structures of ribosomal core particles and reconstitutes as a means of identifying the location of protein L7/L12 within the ribosome. A preliminary reconstruction of 70S tight couples was used as a reference model for determining the orientational angles of particle images of both 70S cores and L7/L12 reconstituted ribosomes. After correction of the particle images for the contrast transfer function (CTF) of the electron microscope and three rounds of refinement, the two final reconstructions had a resolution of 26 Å at the 0.5 correlation cutoff of the Fourier shell correlation (40) (Figure 2). Three-dimensional maps of the L7/L12 reconstituted ribosome (green) and the 70S cores (blue) are shown in Figure 3. As shown by HPLC and mass spectroscopy, the preparations of L7/L12 reconstituted ribosomes and the 70S cores differ only in the four copies of protein L7/L12. This results in a difference of 48 kDa in the 2.5 MDa mass of the entire ribosome. Thus difference mapping is needed to visualize the location of L7/L12. A difference map was calculated between the reconstituted and core particle reconstructions revealing significant difference density in the L7/L12 shoulder region (red). The volume of the observed difference density corresponds to 19 kDa of protein. Since the N-terminal domain is known to anchor L7/L12 to the ribosome and the hinge region is flexible, we propose that the difference density corresponds to four copies of the N-terminal domain (15 kDa) and a portion of the four hinge regions (4 of 6 kDa). We
presume that the C-terminal domains of L7/L12 are not observed in the difference map because of either static or dynamic structural disorder.

Small conformational changes are observed throughout the ribosome, specifically in the regions assigned to L1, L9, the 30S head, and the base of the 50S subunit. The L1 region of the core structure is smaller and less well defined than in the reconstituted structure, consistent with the observation of local structural flexibility in the L1 protein region (4,42). Differences are also observed below the L1 region, where protein L9 has been localized by immune electron microscopy (43). The structure of protein L9 has been determined by a combined use of x-ray crystallography and nuclear magnetic resonance (NMR) (44). Matadeen et al. (4) have fit the atomic structure of L9 within their cryo-EM reconstruction of the 50S subunit and they propose a shift of ~50 Å for this protein in the intact ribosome. We also note conformational changes in the region of the 30S head and neck, which are not surprising since Agrawal et al. (42) have found that the 30S neck is one of the most flexible regions of the ribosome. The 30S head appears to be held closer to the central protuberance of the 50S subunit in the reconstituted ribosome structure.

Our position for the N-terminal region of L7/L12 found by difference mapping (Figure 3) is compatible with the extensive literature that localizes the protein to this region of the ribosome (17). Specifically we find L7/L12 next to the inferred location of L11. The crystal structure of an L11-RNA complex (45) was positioned into the 5 Å resolution crystallographic density map of the Haloarcula marismortui 50S subunit (46). The crystal structure of the L11-RNA complex (45) was also fit in the 11.5 Å resolution cryo-EM density map of the E. coli 70S ribosome (5). Since it is easier to compare two cryo-EM structures, we include our difference map in one orientation (Figure 3B) matching that of a figure in Gabashvili et al. (5). In this view, the elongated density assigned to L11 is observed to fold over the L7/L12 difference density. Our finding of neighboring locations for L7/L12 and L11 is consistent with previous cross-linking experiments that show both the N- and C-terminal domains of L7/L12 are adjacent to L11 (17).
Cryo-electron microscopy of Nanogold-labeled L7/L12 reconstituted ribosomes

Analysis of ribosomes reconstituted with Nanogold-labeled L7/L12 allowed placement of the C-terminal domains. Native L7/L12 lacks cysteine, therefore a mutant form, Cys-89 L7/L12 (28), was used. Derivatization of the single cysteine with Nanogold produced protein that was labeled near the tip of the C-terminal domain, as shown in Figure 4. After the Nanogold reaction, the protein was isolated from unreacted reagent by HPLC and two SDS-PAGE gels were run, one stained for protein and the other stained for Nanogold. The extent of Nanogold modification of L7/L12 was judged to be nearly 100% immediately after isolation of the labeled protein. After storage of the labeled protein for one week, gel analysis indicated that only a negligible percentage (<5%) of the L7/L12 still contained Nanogold. Thus the subsequent steps involved in preparation of reconstituted ribosomes with Nanogold-labeled L7/L12 and purification of 70S ribosomes by sucrose gradient centrifugation and dialysis were carried out as quickly as possible. Cryo-EM grids were prepared on the second day following isolation of the Nanogold-labeled L7/L12 protein. Cryo-EM images of the Nanogold-labeled L7/L12 reconstituted ribosomes indicated that only ~30% of the particles contained Nanogold. Since negative stain EM analysis indicated >90% incorporation of Nanogold-labeled L7/L12, as was found for the wild type L7/L12 reconstitution, we conclude that significant loss of the gold label occurred during preparation and isolation of the Nanogold-labeled 70S ribosomes. Another compounding factor that led to collection of a much smaller cryo-EM data set for the Nanogold-labeled ribosomes than for either the reconstituted wild type or core ribosomes was that the Nanogold-labeled sample was ~10 times less concentrated. Thus for the Nanogold-labeled sample many fewer ribosomes were observed in each digitally collected cryo-electron micrograph (1024 x1024 pixels).

Figure 5A shows examples of cryo-EM particle images, preprocessed in QVIEW (36), of Nanogold-labeled ribosomes. Only particles that clearly showed gold clusters,
visible as intense dark spots, were selected leading to a total data set of 486 particle images. These particle images contained 1 to 4 gold clusters each. Even if there were no loss of the gold label, 4 well separated gold clusters would not have necessarily been seen in each particle image because of the particle orientation. Thus no attempt was made to select for particle images of ribosomes with all 4 possible sites labeled. Orientational angles were determined for the Nanogold-labeled ribosome particle images using the reconstruction of the wild type L7/L12 reconstituted ribosome as a reference. A preliminary reconstruction was calculated from the full Nanogold-labeled ribosome data set. Comparison of the Nanogold-labeled ribosome particle images with projections of the preliminary reconstruction indicated that correct orientational angles were only found for ~65% of the particle images. Given the high probability that some gold label was lost and that not all of the ribosomes in the data set had the same complement of sites labeled, we chose not to refine the incorrect orientational angles, but rather to select the subset of particle images with apparently correct orientational angles. Thus a relatively small set of 317 Nanogold-labeled ribosome particle images was used to generate the final Nanogold-labeled ribosome reconstruction. The resolution of the Nanogold-labeled reconstruction was assessed in two ways, first by calculating a FSC plot between two half-reconstructions and second by calculating a FSC plot between the reconstruction of the full Nanogold data set with the reconstruction of the wild type reconstituted ribosome. Given the small size of the data set it is not surprising that splitting the data set gave a poorer resolution, 60 Å, than the comparison with the wild type reconstruction, which indicated 45 Å resolution. The better resolution estimate seems more correct as filtering the Nanogold reconstruction to 60 Å overly smoothes real structural features that can be observed in the Nanogold reconstruction filtered to 45 Å resolution (Figure 5B-E).

Localization of L7/L12 by Nanogold labeling
Sites of Nanogold density were visualized by subtracting the structure of the wild type L7/L12 reconstituted ribosome from the Nanogold-labeled L7/L12 ribosome reconstruction after filtering both density maps to 45 Å resolution. The difference mapping was repeated with both input density maps filtered to 60 Å resolution and the same Nanogold sites were observed. The Nanogold difference map revealed four clusters of density (yellow) shown together with the density attributed to the N-terminal region of L7/L12 (red) and the core particle reconstruction (blue) in Figure 6. One of the four Nanogold sites, density II, is adjacent to the L7/L12 density (red), while the centers of Nanogold sites I, III, and IV are each about 80-90 Å away. This distance is consistent with the length of the maleimido-Nanogold label (20 Å), plus the length of the C-terminal domain of L7/L12 (35 Å), and a portion of the hinge region (50 Å maximum).

The largest density attributed to Nanogold (labeled I) localizes on the 30S subunit head on the side facing the 50S subunit. The second largest density (labeled II) is elongated and is situated on the body of the 50S subunit below the (red) protein density. Additional Nanogold density is present just below the 50S central protuberance on the side of the L1 shoulder where it is present as two small masses (labeled III), and high on the body of the 30S (labeled IV). Our interpretation of these observations with respect to the location of protein L7/L12 is summarized in the models presented in Figure 7.
Discussion

Location of protein L7/L12 within the E. coli 70S ribosome

The difference map between core particles and reconstituted ribosomes has allowed us to visualize the location of L7/L12 N-terminal domains, which interact strongly with the 50S subunit, while the intense density of the Nanogold label let us mark positions of the mobile C-terminal domains. As illustrated in Figure 7, we postulate that the flexible L7/L12 hinge enables the paired C-terminal domains to localize to at least four discrete positions in the 70S ribosome, although not at all four sites simultaneously. The electron dense Nanogold label has allowed us to capture these partially occupied sites. Evidence for a different conformation, location and function of each dimer is plentiful (22), and each site identified in our work is compatible with biochemical data.

In all panels of Figure 7 one L7/L12 dimer is shown in a conformation in which the hinge bends and contracts to bring the C-terminal domains onto the 50S body, adjacent to the N-terminal domains. The hinge of this dimer could be in a rigid arrangement and may account for the narrow tail of difference density that extends toward Nanogold site II (Figure 6). We postulate that this conformation corresponds to the high affinity site first described by Zantema et al. (47); the domain placement is consistent with localizations by immune electron microscopy (41) and with the cross-linking (e.g. to proteins L10 and L11; (22)) and energy transfer studies with native and mutant proteins (21). Figure 7A-C shows conformations of the second L7/L12 dimer in which the hinge extends into the interface cavity, allowing the C-terminal domains to reach sites that are distant from the (so called) L7/L12 shoulder. The site on the 30S head, Figure 7A, is compatible with the cross-linking to 30S subunit proteins S7 and S14, which is enhanced by elongation factor Tu (22). Figure 7B shows the C-terminal domains located below the central protuberance, in proximity to the peptidyl transferase center, and at a location compatible with the observed cross-links to the 50S subunit proteins L2 and L5 (22,48,49). Finally, Figure 7C shows a
conformation in which the protein extends straight towards the head/body junction of the 30S subunit and the C-terminal domains are at a location compatible with the cross-links to S2 and S3 (50).

Given the flexibility and extended nature of L7/L12, these sites of the L7/L12 C-terminal domains are all within reach of the difference density we attribute to the N-terminal anchors. The variation observed in the size of the four Nanogold density sites may relate to the fact that within a single dimer the relative separation and orientation of the C-terminal domains have been shown to vary (21,51). In our interpretation the four sites identified by Nanogold labeling most likely correspond to alternative L7/L12 conformations, and illustrate the mobility of the C-terminal domains of protein L7/L12 within the ribosome.

Long range interactions and conformational changes in the ribosome

In comparing our reconstructions of the reconstituted and core ribosomes, we noted some conformational changes in the L1 and L9 region, on the opposite side of the ribosome from the L7/L12 shoulder. This is consistent with evidence for a long range interaction between the two sides of the ribosome; e.g. binding of an antibody to protein L9 below the L1 shoulder strongly interferes in translocation factor binding and function that occur in the L7/L12 region (52).

Conformational changes upon subunit association and binding of factors have been reported for cryo-EM (4,6,8,42,53,54) and x-ray (11,46,55) structures. In addition conformational changes associated with tRNA (6,7,56) and mRNA binding (57) and translocation (53) are well documented. The ribosome also alters its conformation in response to changes in divalent cations (58) and during its ordered assembly from RNA and proteins (23). Incorporation of L7/L12 into core particles also appears to cause conformational changes throughout the ribosome as shown by our results.

It has been assumed that all four copies of protein L7/L12 are present in the 50S stalk because electron micrographs of negatively stained 50S particles that lack L7/L12
show no stalk, and reincorporation of the protein restores this structure. This generalization, although challenged as early as 1982 (47), remains prevalent in the literature. Monoclonal antibody labeling studies on isolated 50S subunits have shown that the C-terminal region of L7/L12 can be localized both to the end of an extended stalk and to a site on the body of the 50S subunit (41). Our Nanogold labeling study indicates four sites for the C-terminal domain of L7/L12 in the reconstituted 70S ribosome, two of which are located on the 30S subunit. An extended stalk has only been observed in cryo-EM reconstructions of the ribosome complexed with other molecules, such as tRNAs and elongation factor G (40,53). The absence of an extended stalk in other cryo-EM reconstructions has been explained by the presumed flexibility of the stalk and its ability to fold back onto the body of the 50S subunit (6,10,59). Our difference mapping and Nanogold labeling studies indicate that the two dimers of L7/L12 are able to fold back onto both the 30S and the 50S subunits of the 70S ribosome.

Functional implications of the location of L7/L12

Our results show the hinge regions and C-terminal domains of at least one dimer of protein L7/L12 to be present in the intersubunit cavity, in an area occupied by the elongation factors upon interaction with the ribosome. EF-G has been placed in this part of the ribosome by difference mapping of cryo-EM reconstructions (53,60); the factor interacts with the 50S subunit in the area we associate with the N-terminal domains and the C-terminal domains of the high affinity site (Figure 6, density II). Additional contact of EF-G is to the 30S subunit, at the subunit neck and not far from densities III and IV of Figure 6. Placement of tRNA molecules in cryo-EM (7,56) and x-ray (11) structures shows contact regions on the small subunit in the region of densities I and IV, and density III is very near the peptidyl transferase center of the large subunit. Cross-linking data show that binding of an EF-Tu-tRNA complex alters the frequency of formation of specific links; e.g. cross-linking from Cys-89 to L5 (near density III), L10 and L11 (near density II) and S3 (beside density
IV) are decreased, while links to S7, S14 and S18 (in the 30S head and platform) are increased (22). This suggests movement of the C-terminal domains through the cycle of addition of each amino acid, and raises the possibility that additional stable sites for this element of the protein exist at different stages of the translation cycle.

In conclusion, we have localized the N-terminal domains of protein L7/L12 within the E. coli 70S ribosomes on the body of the large ribosomal subunit, and used Nanogold labeling to identify allowed sites for the C-terminal domains. Our results provide further evidence for the mobility of the C-terminal domains of the protein within the ribosome, as well as for the conformational flexibility of the ribosome itself.
Acknowledgements

We thank Dr. Kim Faull for the mass spectrometry and Dr. Sergey Ryazantsev for help in holey grid preparation. We also thank Dr. J. Frank and R. Grassucci for sharing their sample preparation expertise, and Drs. Charles Chiu, Jamie Schlessman, Lawrence Kong, and Dana Haley for useful discussions and help in image analysis and reconstruction. This work was supported by USPHS NIH Grants GM51195 (to DGG) and GM 17924 (to RTT), and by National Science Foundation Grant MCB-9722353 (to PLS).
References


Figure Legends

Figure 1. HPLC of ribosomal protein extracts. (A) Extract from 300 pmoles of 70S ribosome tight couples. (B) Extract from 300 pmoles of 70S cores. (C) Extract from 25 pmoles of reconstituted ribosomes.

Figure 2. Resolution assessment of cryo-EM reconstructions. Fourier shell correlation function between two independent reconstructions of L7/L12 reconstituted ribosomes (dark continuous line) and 70S cores (gray continuous line) shown with the 3σ threshold curve (broken line).

Figure 3. Two ribosome reconstructions and the difference map filtered to 26 Å resolution. Green: L7/L12 reconstituted ribosomes. Blue: 70S cores. Red: difference map between reconstituted ribosome and core reconstructions. The L1 and L11 regions as well as the central protuberance (CP) are labeled on the reconstituted ribosome reconstruction. (A) A view toward the 50S side. (B) A rotated view to roughly correspond to the orientation of the 50S subunit as shown in Figure 7 (bottom) of reference (5). The two arrows denote a protein and a rRNA fragment modeled by Gabashvili et al. (5). (C) A view from the top with the 30S on the left.

Figure 4. Structure of ribosomal protein L7/L12 and a Nanogold derivative. (A) Diagram of the domain structure of L7/L12. (B) Crystal structure of the C-terminal domains of the L7/L12 dimer showing the sites of the Cys-89 mutation. (C) Schematic drawing of the maleimido-Nanogold complex bound to protein. (D) Diagram of L7/L12 labeled with Nanogold.
Figure 5. Cryo-electron microscopy of Nanogold-labeled L7/L12 reconstituted ribosomes. (A) Raw images, preprocessed in QVIEW, of Nanogold-labeled ribosomes taken at -1.5 µm defocus. (B-E) Reconstruction of the Nanogold-labeled reconstituted ribosome filtered to 45 Å resolution. (B) A view toward the 50S side. (C) A side view with the 30S on the left. (D) A view toward the 30S side. (E) A top view with the 30S on the left.

Figure 6. Localization of L7/L12 within the 70S ribosome. Yellow: Nanogold density (isocontoured at 5σ) from the difference map between the Nanogold-labeled L7/L12 reconstituted ribosome and the wild type L7/L12 reconstituted ribosome reconstructions (both filtered to 45 Å resolution). Red: density difference attributed to L7/L12 from Figure 3. Blue: 70S cores from Figure 3. In the left panels the 70S core provides a reference for the locations of the difference densities within the ribosome; in the right panels the core is not shown so that the full extent of the L7/L12 density (red) and the four gold density regions are visible. (A) A side view with the 30S on the left and the 50S on the right. (B) A top view, as in Figure 3C, with the 30S on the left.

Figure 7. Models of allowed conformations of L7/L12 within the 70S ribosome. All panels show the same conformation for one L7/L12 dimer with the C-termini at the presumed high affinity site on the 50S subunit, corresponding to the Nanogold label site II in Figure 6. Three conformations are shown for the C-termini of the second L7/L12 dimer corresponding to the Nanogold label sites I (A), III (B) and IV (C). The ribosome is oriented with the 30S on the left and the 50S on the right.
Cryo-electron microscopic localization of protein L7/L12 within the Escherichia coli 70S ribosome by difference mapping and Nanogold labeling
Luisa Montesano-Roditis, Dohn G. Glitz, Robert R. Traut and Phoebe L. Stewart

*J. Biol. Chem.* published online January 30, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M008430200](http://doi.org/10.1074/jbc.M008430200)

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