Cross-linking of Selected Residues in the N- and C-terminal Domains of Escherichia coli Protein L7/L12 to Other Ribosomal Proteins and the Effect of Elongation Factor Tu*

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Five different variants of protein L7/L12, each with a single cysteine substitution at a selected site, were produced, modified with [125I-4-[N-(p-azidosalicylamido)-butyl]-3-(2'-pyridyldithio)propionamide, a radiolabeled, sulfhydryl-specific, heterobifunctional, cleavable photocross-linking reagent that transfers radiolabel to the target molecule upon reduction of the disulfide bond. The proteins were reconstituted with core particles depleted of wild type L7/L12 to yield 70 S ribosomes. Cross-linked molecules were identified and quantified by the radiolabel. No cross-linking of RNA was detected. Two sites in the dimeric N-terminal domain, Cys-12 and Cys-33, cross-linked strongly to L10 and in lower yield to L11 but to no other proteins. The three sites in the globular C-terminal domain all cross-linked strongly to L11 and, in lower yield, to L10. Weaker cross-linking to 50 S proteins L2 and L5 occurred from all three C-terminal domain locations. The 30 S ribosomal proteins S2, S3, S7, S14, S18 were also cross-linked from all three of these sites. Binding of the ternary complex [14C]Phe-tRNA - elongation factor Tu-GDP-kirromycin increased labeling of L2, L5, and all of the 30 S proteins. These results imply the flexibility of L7/L12 and the transient proximity of three surfaces of the C-terminal domain with the base of the stalk, the peptidyl transferase domain, and the head of the 30 S subunit.

The Escherichia coli ribosomal protein, L7/L12, is the most extensively investigated representative of the small, four-copy, dimeric acidic proteins that are found in large ribosomal subunits of all organisms and exist as a conserved quaternary structural element in which two dimers are integrated into the ribosome through binding to a common anchoring protein (1–3). One or both of the L7/L12 dimers form a conspicuous morphological feature on the ribosome known in E. coli as the L7/L12 stalk (4). The proteins can be simply and selectively removed from and reconstituted into the ribosome (5), a property that greatly facilitates experiments of the type reported here in which genetically and biochemically modified proteins replace the wild type.

The L7/L12 polypeptide is composed of two distinct organized structural domains linked by a flexible hinge (6, 7), as summarized in Fig. 1. The elongated, helical N-terminal domain, residues 1–33, is responsible for dimer interaction (8), and the globular C-terminal domain, residues 53–120, is necessary for factor binding (9–11). The C-terminal domains can be cross-linked to each other in different orientations yet retain full functional activity in supporting polypeptide synthesis (12). Flexibility of L7/L12 has been demonstrated in solution by NMR (14, 15), by electron microscopy (16), and with fluorescence probes attached to the C-terminal domains (13, 17, 18).

Immunoelectron microscopy with monoclonal antibodies (19) directly showed the presence of the C-terminal domain at the tip of the stalk and the N-terminal domain at the base of the stalk. This was consistent with earlier demonstrations that the N-terminal domain was responsible for binding of the full-length L7/L12 to L10 and to the ribosome (9, 10). It was shown that one dimer per particle was sufficient to form a visible stalk (20), despite earlier studies with polyclonal antibodies that had suggested that both dimers were present in the stalk (21). Different studies indicated that the presence of one L7/L12 dimer on the body of the 50 S particle in an extended conformation directed toward the central protuberance (22, 23). Additional evidence that a C-terminal domain can occupy a location not only extended across the body of the ribosome but also near the base of the stalk came from cross-linking between a predetermined location in the C-terminal domain, Cys-89, and Cys-70 of L10 (24, 25) and also from hinge deletion studies (26, 27). A different heterobifunctional reagent, APDP1 showed a cross-link between Cys-89 and L11 and also with L10 in a lesser extent near the EF-G binding site near the base of the stalk (28). The site-specific cross-linking experiments led to the proposal of a possible “bent” conformation for one of the dimers in which the C-terminal domain could lie on the body of the subunit near the N-terminal domain at the base of the stalk. We asked the question whether there was a preferred surface of the C-terminal domain that made these contacts and designed cysteine probe sites on other faces of the C-terminal domain. In addition we investigated whether any protein other than those at the base of the stalk made contact with the C-terminal domain. Previous experiments with 2-iminothiolane showed contact between an undefined lysine of L7/L12 with proteins L2 and L5 (22, 29). Two probe sites in the N-terminal domain were also tested. The present approach permits us to define which domain makes these distant contacts. The effect of elongation factors on the pattern of cross-linking was also examined.

EXPERIMENTAL PROCEDURES

APDP and Iodobeads were products of Pierce. 125I was procured from Amersham Life Science, Inc. All other chemicals were of reagent grade.

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* The abbreviations used are: APDP, N-[4-(p-azidosalicylamido)-butyl]-3-(2'-pyridyldithio)propionamide; EF-G, elongation factor G; GMP-P(NH)P, guanyl-5'-yl imidodiphosphate.

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L12[Cys63], L7/L12[Cys89], and L7/L12[Cys99] are designated as SDS-PAGE. The mutant proteins L7/L12[Cys12], L7/L12[Cys33], L7/L12[Cys63], L7/L12[Cys89], L7/L12[Cys99], and L7/L12[Cys99] are designated as Cys89, Cys63, Cys33 variants as described previously (26). The two additional amino acid substitutions, Ala-12 to Ser-33 from N-terminal domain (A) and the location of Ala-63, Ser-89, and Ser-99 in the x-ray crystallographic structural domain (B). Rasmol molecular viewer software is used for the display of the structure. NTD, N-terminal domain; CTD, C-terminal domain, Res., residue.

Preparations of Ribosomes and Ribosomal Core Particles—Ribosomes were prepared from midlog culture of E. coli MRE600 as described earlier (30). The ribosomes used in these experiments were more than 90% tight couple population as determined by sucrose gradient centrifugation. Core ribosomes completely lacking L7/L12 (P0 cores) protein were prepared from 70 S ribosomes following the method of Ref. 5 with modifications as described (21). They were stored in aliquots at –86 °C.

Construction, Overexpression, and Purification of Cysteine-substituted Variants of L7/L12—Wild type L7/L12 lacks cysteine. The preparation of the Cys-89, Cys-63, Cys-33 variants was as described previously (26). The two additional amino acid substitutions, Ala-12 to Cys-99 were generated using the site-specific oligonucleotide-directed mutagenesis system from Amersham (12). The purity of the final preparations was estimated to be at least 95% by SDS-PAGE. The mutant proteins L7/L12[Cys12], L7/L12[Cys33], L7/L12[Cys63], L7/L12[Cys89], and L7/L12[Cys99] are designated as Cys12, Cys33, Cys63, Cys89, and Cys99.

Labeling of APDP with 125I—APDP was labeled with 125I using Iodobeads™ as described previously (28). The specific radioactivity of 125I was adjusted to 10–20 Ci/mmol by adding cold sodium iodide. Radioiodinated APDP was purified by TLC on silica plates (Silica Gel 60 F254; Merck) in chloroform/benzene/ethyl acetate/acetic acid (10:10:10:1). The plate was exposed for a few seconds with Kodak X-Omat film, followed by fluorography. The exposure times were from 3 to 14 h depending on the amount of radioactivity in the gels. The instrument has a broad range of line-sensitivity screens of a Molecular Imager™ System (GS-250, Bio-Rad) for the complete removal of the reducing agent, and then reconstituted individually with each of the five different radiolabeled Cys variants as described previously (28). Excess, or unbound L7/L12 was removed by centrifugation of the particles through a 10% (w/v) sucrose cushion in the same buffer (without 2-mercaptoethanol) for 5 h at 58,000 rpm at 4 °C in a Beckman Ti65 rotor. The reconstituted ribosomal pellet was resuspended in the same buffer (without the reducing agent) at a concentration of 5 mg/ml. Typically, reconstituted 70 S ribosomes in different experiments had between 2.9 and 3.7 copies of 125I[azidophenyl]thio-L7/L12Cys. It had been shown previously that modification with APDP had no inhibitory effect on ribosomal activity in a poly(U)-dependent polyphenylalanine synthesis assay in which 15–20 Phe residues/ribosome were polymerized (28).

Cross-linking and Sample Processing—Cross-linking was initiated by irradiation of the reconstituted ribosome samples kept in standard L.5 ml Eppendorf tubes at 4 °C for 5 min with a UV light source (Black Ray model B-100A, Fisher; UV light >300 nm). The source was mounted to provide a vertical beam of light centered over an opened sample tube at a distance of 10 cm. After the cross-linking reaction, samples were made 1% with 2-mercaptoethanol and incubated at 37 °C for 1 h to cleave the disulfide bond in the cross-link bridge. For final identification, 50 pmol of radioactive ribosomes were mixed with 125 pmol of cold ribosomes in a buffer containing 1% of 2-mercaptoethanol. The total protein was extracted with 66% acetic acid (31), and the extracted proteins were extensively dialyzed against 6% acetic acid at 4 °C and lyophilized. Samples of approximately 140 μg were dissolved in 25 μl of sample buffer containing 20 mM Bio-Tris acetate (pH 5.7), 8 M urea, and 1% 2-mercaptoethanol and analyzed by two-dimensional acid/urea gel electrophoresis (32). The gels were stained with Coomassie R-250 and dried on Whatman 3MM paper.

Analysis of Cross-linked Ribosomal Subunits by Sucrose Gradient Centrifugation—Ribosomes containing L7/L12Cys63 modified with 125I-APDP were analyzed by sucrose gradient centrifugation before photocross-linking, after cross-linking and reduction, in the absence of elongation factors, and in the presence of EF-Tu ternary complexes containing GMP-P(NH)P or GDP. In these experiments, excess L7/L12 used for reconstitution was not removed. Each aliquot contained 4 A260 units of 70 S ribosomes and 644,000 cpm of the 125I-APDP radiolabel. Centrifugation was carried out in an SW-50 rotor for 12 h at 25,000 rpm. The gradient buffer from 5–20% sucrose contained 1 mM MgCl2, 100 mM KCl, 10 mM Tris-HCl (pH 7.2), and 0.2% 2-mercaptoethanol. Fractions (0.45 ml) were collected, and radioactivity were determined for each. The relative amount of radiolabel attached to ribosomal particles is difficult to quantify accurately due to the presence of the preponderant fraction at the top of the gradient. Approximately 2% of the label was found in the ribosome fractions, a yield consistent with nitrene-generating cross-linking reagents (33). For this reason it was impossible to compare quantitatively the different variants.

Identification of Cross-linked Proteins and Quantification of Cross-linking Patterns—Stained and dried gels were exposed with high sensitivity screens of a Molecular Imager™ System (GS-250, Bio-Rad) followed by quantitative analysis of the screen according to the procedures recommended by the manufacturer. The images were processed and quantified using Molecular Analyst™ software (Bio-Rad, Version 1.1.1). The exposure times were from 3 to 14 h depending on the amount of radioactivity in the gels. The instrument has a broad range of linearity, and all images were within this response range. The image size on the computer monitor was set to represent the actual size of the stained gel. Qualitative identification of cross-linked proteins was performed by superimposition of the patterns of radioactivity with a transparency of
Coomassie Blue-stained gel pattern of total protein. For quantification, the positions of all the protein spots corresponding to the stained pattern were given elliptical outlines on the computer image. Each ellipse was centered on the most intensely stained part of each spot, and its size was set to minimize overlap with nearby proteins. The area of the ellipse for each individual protein was kept constant for that protein throughout all experiments. The quantification was performed according to the manufacturer's recommendations. The volume analysis option of the software was used for quantification. The numbers generated by the software represent the number of counts accumulated by the irradiation-sensitive screen of the Molecular Imager in the selected, outlined areas for each protein. To determine the background, the same
procedures were followed for samples identical in every way, except that they were not irradiated. Background values were 5–10% irradiated values. The intensity of labeling for each individual protein was corrected for background and plotted relative to the full scale established from the most highly labeled protein on each gel, L7/L12 itself for the Cys99, Cys89, Cys63, and Cys33 variants and L10 for the Cys12 variant. This normalization facilitated comparison of the distribution of labeling among different proteins in different experiments.

Cross-linking in the Presence of Elongation Factors—Samples of 70 S P0 cores reconstituted with radiolabeled L7/L12 Cys-variant proteins were incubated with EF-G under two different conditions. In a total volume of 100 μl containing 10 mM Tris-HCl (pH 7.4), 10 mM NH4Cl, and 10 mM magnesium acetate, 50 pmol of ribosomes were incubated with 100 pmol of EF-G either in the presence of 30 mM fusidic acid and 1 mM GTP or with 1 mM GMP-P(NH)P at 0 °C for 30 min (34). For experiments with EF-Tu, samples containing 50 pmol of reconstituted 70 S ribosomes in buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 80 mM KCl, and 80 mM NH4Cl were irradiated, and the distribution of cross-linked proteins was analyzed as described above. Background values were 5–10% irradiated. That the major target of irradiation was the 50 S subunit is to be expected from the location of L7/L12 and, as seen later, from the fact that a major photoproduct is L7/L12, itself due to transfer from one L7/L12 labeled at Cys-63, the location that consistently gave the highest overall level of labeling. Panel A shows that there is radioactivity coincident with the 50 S peak and possibly with the 30 S subunit. The subunit specificity and UV dependence of radiolabeling was analyzed by sucrose gradient centrifugation. The samples were reduced, and the gradient contained reducing agent. Any labeling of subunits indicates transfer of radiolabel from L7/L12 to a different neighboring location. Fig. 2 shows these results for L7/L12 labeled at Cys-63, the location that makes it difficult to estimate any label in the 30 S subunit. The A260-absorbing material at the top of the gradient is due to disulfide-bridged cross-links. Subsequent reduction breaks the bridge, with the concomitant transfer of label to the target. The subunit specificity and UV dependence of radiolabeling was analyzed by sucrose gradient centrifugation. The samples were reduced, and the gradient contained reducing agent. Any labeling of subunits indicates transfer of radiolabel from L7/L12 to a different neighboring location. Fig. 2 shows these results for L7/L12 labeled at Cys-63, the location that consistently gave the highest overall level of labeling. Panel A shows that there is radioactivity coincident with the 50 S peak and possibly with the 30 S subunit.
the top of the gradient. There was no label coincident with the 23 and 16 S ribosomal RNA peaks. This was the case for all five variants (results not shown). Accordingly, characterization of the radiolabeled ribosomal proteins was pursued.

Fig. 3 shows analysis of ribosomal proteins by two-dimensional gel electrophoresis. Panel 3A shows the stained gel of 70 S proteins. The gel system separates most 70 S ribosomal proteins with good resolution, and this is the case for the labeled proteins. Panel 3B shows the distribution and identities of radiolabeled proteins with the $^{125}$I-APDP probe attached at Cys-63, the probe location that gave maximal labeling of proteins other than L7/L12 itself. The ellipsoids indicate how the Molecular Imager analysis was conducted as detailed under “Experimental Procedures.” Proteins L7/L12, L10, L11 are the major labeled proteins. Proteins L2 and L5 are labeled to a less, but clearly significant extent. In addition to 50 S proteins, 30 S ribosomal proteins S2, S3, S7, S14, and S18 are clearly labeled. Protein S4 is visible in this gel but is generally found only at a very low level and only with Cys63. Only a low level of labeling occurred in the absence of irradiation, due to difficulty in completely excluding stray light, as indicated by the sucrose gradient analysis. Only L7/L12 itself was labeled when irradiation was performed before reconstitution. The result rules out labeling by disulfide interchange. When modified, L7/L12 was irradiated after mixing with 30 S subunits, and a low level of S2 labeling occurred in addition to the homolabeling.

Panels C, D, E, and F show the patterns of labeling for variants Cys89, Cys99, Cys12 and Cys33, respectively. The patterns for the C-terminal variants Cys89 and Cys99 are qualitatively similar to Cys63. The patterns for the N-terminal variants Cys12 and Cys33 are notably different. Labeling is confined to the L7/L12, L10, L11 regions, and there is no labeling of 30 S subunit proteins.

Equal amounts of total ribosomal protein from ribosomes reconstituted with the different C-terminal domain variants radiolabeled with the same specific activity were analyzed. When homolabeling was excluded there were differences in the overall yield of labeling of target proteins for the three variants. Cys63 gave the highest yield, and Cys99 gave the lowest.

The Molecular Imager was programmed to quantify the radiolabel present in each protein spot relative to the total detected on the gel. The results for all five variants are shown in Fig. 4. The height of the bars provides a linear depiction of the relative distribution of the extent of labeling among the total proteins. The 10 proteins listed qualitatively from the gels shown in Fig. 3 appear as bars of different heights. For all of the variants except Cys12, L7/L12 itself most heavily labeled species; therefore, the software was programmed to normalize each protein to L7/L12, taken as 1 in the bar graph. The extent of homolabeling (transfer of label to L7/L12 itself) was compared for each variant in the free and ribosome-bound state. Homolabeling in solution for the two N-terminal domain variants was much greater than for the C-terminal domain variants, with Cys63 distinctly lower than Cys89 and Cys99. Ribosome binding had no effect on the C-terminal domain sites but greatly lowered the Cys12 homolabeling and reduced Cys33 homolabeling (data not shown).

Cross-linking from the N-terminal domain sites to proteins other than L7/L12 was limited to proteins L10 and L11, with L10 the strongest. For Cys12, L10 labeling exceeded that of L7/L12. The labeling of L10 from both Cys12 and Cys33 greatly exceeded L10 labeling from any of the C-terminal domain sites. Protein L11 was labeled to a lesser extent than L10, and the probe from Cys33 gave more L11 labeling than Cys12. The different labeling patterns for the different Cys residues again reinforces the specificity of the photochemical reaction and the absence of labeling by chemical disulfide interchange. Cross-linking from the three C-terminal domain sites was strongest.
to L11, a result reported previously for Cys99 (28). Cross-linking to L10 was lower than to L11. L11 was labeled more from the C-terminal domain sites than from the N-terminal domain sites; the converse was the case for L10. Homolabeling was of approximately the same magnitude as L10 and L11 (results not shown because of the normalization). Weaker, but significant labeling of L2 and L5, cross-links found previously with 2-iminothiolane (22), was detected from all three C-terminal domain sites. The C-terminal domain sites cross-linked to certain 30 S proteins, more strongly to S3, but significantly to S2, S7, S14, and S18, the extent of labeling being comparable to that of L2 and L5.

Effect of Elongation Factors on the Cross-linking Pattern—The distribution of cross-links for each of the five L7/L12 variants was determined in the presence of EF-G-GDP-fusidic acid, EF-G-GMP-P(NH)P and the EF-Tu-GMP-P(NH)P-Phe-tRNA, and the EF-Tu-GDP-kirromycin ternary complexes. Neither of the EF-G complexes gave any consistent, major alteration in the distribution of cross-links (results not shown). The EF-Tu complexes made with Cys63 subunits were first analyzed by sucrose gradient centrifugation and are shown in Fig. 5. The kirromycin complex shows a lower extent of 50 S labeling than the GMP-P(NH)P complex and resembled that with no factor present. The results were the same for the other variants (results not shown). The pattern of protein labeling was analyzed and quantified as before. These results with the EF-Tu ternary complexes are shown in Fig. 6, which compares the extent of labeling of the 10 major proteins in the absence and presence of the two EF-Tu complexes. Because there were significant differences in homolabeling, results were normalized to L11, taken as 1. Little difference can be discerned between the kirromycin complex and the pattern with no factor present. For the GMP-P(NH)P complex, there is a consistent increase in the labeling of L2, L5, and small subunit proteins S2, S3, S7, and S14. In addition, the homolabeling is increased.

**DISCUSSION**

Fig. 7 shows the location on the ribosome of the proteins cross-linked from the various sites in L7/L12. The evidence for the location of 50 S cross-linked proteins L10, L11, L2, and L5 has been presented previously (40). The locations of the 30 S proteins is taken from the immuno-electron microscopy studies of Lake and co-workers (41) and Stöffler-Meilicke et al. (42, 43). The 30 S subunit is shown with the platform on the left, opposite the L7/L12 stalk. All of the 50 S proteins labeled as well as S7 and S18 are on the subunit interface surface of the subunit; S2, S3, and S14 are on the exposed head and neck of the 30 S subunit. The Roman numbers indicate alternative locations in the 50 S subunit for the C-terminal domain of L7/L12 as determined by immuno-electron microscopy and protein cross-linking (40). The letter A indicates a fourth site, implied by the results presented here. The curved arrows suggest movements or conformations of L7/L12 consistent with cross-linking to the target proteins.

The labeling of L11 and L10 from the two sites selected in the N-terminal domain is consistent with the role of the N-terminal domain in binding directly to L10 and the established proximity of L10 and L11 (44). In the C-terminal domain, Cys63 is maximally exposed and more distant from the N-terminus than Cys99, and its formation of cross-links in highest yield is consistent with this. Nevertheless, all three probe locations in the C-terminal domain label L11, and to a lesser extent L10, defining site II. This reinforces the conclusion of a bent conformation for one of the dimers, facilitated by the flexible hinge (28). The labeling from all three residues, 63, 89, and 99 implies that there is no preferred tight interaction of a single C-terminal domain with L10 and L11 in a defined orientation, since it is unlikely that all three regions could simultaneously make the necessary contacts. The labeling from all three sites is more likely facilitated by the presence of two C-terminal domains at site II and their capacity to retain functional activity even when locked in disparate orientations by zero-length disulfide cross-linking (12). The reagent used here maximally extends 21 Å, which also plays a role in facilitating proximity from all three residues. The labeling of proteins L2 and L5 in peptidyl transferase site (site III) confirms earlier results with 2-iminothiolane (22) and defines the C-terminal domain as the relevant domain. In that study, L7/L12-L5 cross-link was identified in 50 S but not 70 S ribosomes, and it was suggested that competition for L5 lysines by 30 S interface proteins could account for this finding. The present result with 70 S ribosomes implies that 30 S binding does not block access across the interface cavity.

The cross-linking of 30 S proteins was an unexpected result, since none had been detected with 2-iminothiolane (22). The lysine specificity of that approach versus the nonspecificity of the photocross-linking used here may explain the different results. Proteins S2, S3, and S14 are clustered on the exterior of the head of the 30 S subunit on the same side of the 70 S
particle as the L7/L12 stalk at site A. The location of this group of proteins coincides nearly perfectly with that determined by immunoelectron microscopy for EF-Tu (45, 46). Previous studies indicated that the conformation of L7/L12 is modulated by interaction of elongation factors with the ribosomes and depends on hydrolysis of GTP (47). Movement of the flexible stalk dimer could bring the C-terminal domain near these proteins. The results suggest the participation of the C-terminal domain of L7/L12 in EF-Tu binding at the R (recognition) site and thus imply a role for L7/L12 in factor binding at a site other than the site at the base of the stalk where EF-G binds. The stimulation of labeling of 30 S proteins by EF-Tu is consistent with a conformational change that brings L7/L12 in closer proximity to sites on the small subunit. The proteins S7 and S18 are far from each other and far from the S2-S3-S14 cluster. Protein S7 is located near L5 and L2, and the extended orientation (site III) can also account for S7 labeling. The labeling of S18 is not easily explained in the static model depicted. The increase in S18 labeling caused by EF-Tu suggests a conformational change that brings the C-terminal domain near these proteins. The probe does not sweep out a swath of labeled site at the base of the stalk where EF-Tu in the 30 S site and be involved in its movement.

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**Ribosomal Location of L7/L12 Domains by Photocross-linking**

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