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The C-Terminal Domain of *Escherichia coli* Ribosomal Protein L7/L12 Can Occupy a Location near the Factor-Binding Domain of the 50S Subunit As Shown by Cross-Linking with *N*-[4-(*p*-Azidosalicylamido)butyl]-3-(2′-pyridyldithio)propionamide†

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ABSTRACT: All large ribosomal subunits contain two dimers composed of small acidic proteins that are involved in binding elongation factors during protein synthesis. The ribosomal location of the C-terminal globular domain of the *Escherichia coli* ribosomal acidic protein L7/L12 has been determined by protein cross-linking with a new heterobifunctional, reversible, photoactivatable reagent, *N*-[4-(*p*-azidosalicylamido)-butyl]-3-(2′-pyridyldithio)propionamide. Properties of this reagent are described. It was first radiolabeled with ^{125}I and then attached through the formation of a disulfide bond to a unique cysteine of L7/L12, introduced by site-directed mutagenesis at residue 89. Intact 50S ribosomal subunits were reconstituted from L7/L12-depleted cores and the radiolabeled L7/L12Cys89. Irradiation of the reconstituted subunits resulted in photo-cross-linking between residue 89 and other ribosomal components. Reductive cleavage of the disulfide cross-link resulted in transfer of the ^{125}I label from L7/L12Cys89 to the other cross-linked components. Two radiolabeled proteins were identified, L11 and L10. The location of both of these proteins is well established to be at the base of the L7/L12 stalk near the binding sites for the N-terminal domain of both L7/L12 dimers, and for elongation factors. The result indicates that L7/L12 can have a bent conformation bringing the C-terminal domain of at least one of the L7/L12 dimers at or near the factor-binding domain. The cross-linking method with radiolabeled *N*-[4-(*p*-azidosalicylamido)butyl]-3-(2′-pyridyldithio)propionamide should be applicable for studies of other multicomponent complexes that can be reconstituted.

Ribosomal protein L7/L12 of *Escherichia coli* is the most extensively investigated representative of the small, four-copy, dimeric acidic proteins that are found in large ribosomal subunits of all organisms. In eubacteria, eukaryotes, and archaea these proteins always exist as a conserved quaternary structural element in which two dimers are integrated into the ribosome through binding to a common anchoring protein (Casiano et al., 1990; Liljas, 1982; Uchiumi, 1987). One or both of the dimers forms a conspicuous morphological feature on the ribosome known in *E. coli* as the L7/L12 stalk. These proteins have been well studied with regard to defining protein structure–function relationships. They can be simply and selectively removed from and restored to the ribosome with the concomitant loss and regain of activity (Hamel et al., 1972). In both eubacteria and eukaryotes the proteins are required for the binding of elongation factors, and also initiation and termination factors. They represent a major example of ribosome function in which specific proteins play a clearly defined and perhaps dominant role. Protein L7/L12 of *E. coli* is composed of two distinct structural domains separated by a putative flexible hinge (Leijonmarck et al., 1981): an elongated, helical N-terminal domain, residues 1–36, that is responsible for the dimerization of the protein, and a globular C-terminal domain, residues 53–120. The high-resolution crystal structure of the C-terminal domain of the protein has been determined (Leijonmarck et al., 1987). It is this element that has been implicated in factor binding, since truncated L7/L12 fragments that lack the C-terminal domain fail to support protein synthesis even though they bind to the ribosome (Koteliansky et al., 1978; Van Agthoven et al., 1975), and antibodies to the C-terminal domain inhibit the binding of elongation factors as well as protein synthesis (Sommer et al., 1985). Determining more precisely the ribosomal location of the functionally important C-terminal domains has been a goal of work in this laboratory.

The location of both dimers of L7/L12 on the ribosome is unclear, and the determination is made more difficult insofar as the two dimers may have different locations (Möller et al., 1983; Olson et al., 1986; Zantema et al., 1982) and because one or both dimers are mobile (Cowgill et al., 1984; Gudkov et al., 1982; Tritton, 1978) and may undergo major conformational changes during the ribosome cycle. Cross-links between L7/L12 and proteins distant from the L7/L12 stalk have been identified (Redl et al., 1989; Traut et al., 1983). Electron microscopy of intact 50S particles and those from which L7/L12 had been selectively removed led to the identification of the L7/L12 stalk (Strycharz et al., 1978). Immune electron microscopy with polyclonal antibodies suggested that both L7/L12 dimers were located in the stalk with all four C-terminal domains at the tip [reviewed by Liljas (1982)]. The binding site for EF[1]-G is located on the body of the ribosome near the base of the stalk (Girshovich et al.,

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[1] Abbreviations: APDP, *N*-[4-(*p*-azidosalicylamido)butyl]-3-(2′-pyridyldithio)propionamide; APT-L7/12Cys89, (azidophenyl)thio-L7/L12Cys89; SDS, sodium dodecyl sulfate; DPDPB, 1,4-bis[3-(2′-pyridyldithio)propionamido]butane; EF, elongation factor; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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1981). The apparent contradiction between the involvement of the C-terminal domains in factor binding and their location at the tip of the stalk appeared resolved by the finding of a secondary binding site for C-terminal specific monoclonal antibodies at the base of the L7/L12 stalk near the site where EF-G binds. We have attempted to provide additional evidence for the proximity of the C-terminal domain to the factor binding site on the body of the 50S subunit.

Cysteine site-directed mutagenesis was used to substitute serine 89 with cysteine 89 in the C-terminal domain of L7/L12 in order to investigate the interactions or contacts of this predetermined location with other ribosomal components, utilizing the unique chemical reactivity of the introduced cysteine sulfhydryl group. The protein, L7/L12Cys89, was overproduced, purified, and shown to reconstitute normally into 50S subunits without any deleterious effect on ribosome function (Zecherle et al., 1992). In an initial study it was shown that the homobifunctional, sulfhydryl-specific cross-linking reagent, 1,4-bis[3-(2′-pyridyldithio)propionamido]-butane (DPDPB), formed a cross-link between L7/L12Cys89 and Cys70 of L10, the protein that anchors the N-terminal domain of L7/L12 to the ribosome. We report here results with another bifunctional cross-linking reagent, *N*-[4-(*p*-azidosalicylamido)butyl]-3-(2′-pyridyldithio)propionamide (APDP). One functional group of the reagent is specific for sulfhydryls and could be used to modify L7/L12Cys89 prior to reconstitution into 50S subunits; the second functional group is photoactivated to form a nitrene that is able to react nonspecifically with neighboring ribosomal components. The reagent offers two advantages that facilitate identification of components that become cross-linked to L7/L12. First, it can be radiolabeled by ^{125}I in the aromatic ring containing the azido functional group; second, the cross-bridge contains a disulfide bond with the consequence that upon reduction the radiolabel is transferred from L7/L12 to the proximal target component(s).

MATERIALS AND METHODS

Reagents. APDP was a gift of Dr. E. Fujimoto of the Pierce Chemical Co. and was stored in a vacuum desiccator over Drierite at 4 °C. HPLC-grade acetonitrile was from Baker. Ultrapure urea was from ICN. Acrylamide and *N,N′*-methylenebis(acrylamide) were from Eastman. Iodobeads were from Pierce Chemical Co. All other chemicals were reagent grade or better.

Preparation of Ribosomes. 50S ribosomal subunits were prepared from mid-log phase *E. coli* strain MRE600 as previously described (Kenny et al., 1979). The subunits were stored at −70 °C in 10 mM Tris·HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol.

Preparation of Ribosomes Lacking Proteins L7/L12. L7/L12 was selectively extracted from 50S ribosomal subunits using the method of Hamel et al. (1972) with modifications as described by Tokimatsu et al. (1981). Extraction was performed at 0 °C and contained 2 mg/mL ribosomes, 10 mM Tris·HCl, pH 7.2, 150 mM NH_4Cl, 10 mM $MgCl_2$, 3.5 mM 2-mercaptoethanol, and 50% (v/v) ethanol. The protein was also extracted from 70S ribosomes as described (Hamel et al., 1972). The ribosomes were pelleted at low speed (8000 rpm in a Sorvall SA-600 rotor at 4 °C), extracted a second time, and collected again by centrifugation at low speed. The final pellet was resuspended at 1 mg/mL in 10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol and stored at −70 °C in small portions.

Preparation of L7/L12Cys89. L7/L12Cys89 was overproduced from a plasmid in *E. coli* and purified by preparative isoelectric focusing in the presence of urea as described (Zecherle et al., 1992).

Spectrophotometric Characterization of APDP. APDP is not soluble in aqueous solutions and must be dissolved in an organic solvent, such as acetonitrile, before use. The reagent is soluble up to approximately 0.5 mM in aqueous buffers when diluted from a concentrated (10 mM) stock solution in acetonitrile. A 10 mM stock of APDP (formula weight 446.55) in acetonitrile was diluted into acetonitrile to give a final concentration between 10 and 50 μM, and the absorbance was measured from A_{200} to A_{350}. The samples were irradiated for various times at either 254 or 366 nm (Mineralight hand-held lamp) at a distance of 1 cm from the light source and rescanned. A sample of APDP (50 μM) in 50 mM $NaPO_4$, pH 8.0, 5% (v/v) acetonitrile was reduced with 50 mM 2-mercaptoethanol for 30 min at 37 °C and scanned from A_{200} to A_{450}.

Labeling APDP with ^{125}I. The presence of a hydroxyl group at the 2 position of the aromatic ring of APDP allows the compound to be labeled with ^{125}I, primarily at position 3, between the azido and hydroxyl groups, but possibly also at position 5, para to the OH (Ed Fujimoto, personal communication, Pierce Chemical Co.). Other bifunctional reagents containing aryl azide moieties have been similarly radiolabeled before use in cross-linking (Ji, 1983; Shepard et al., 1988). APDP was radiolabeled by solid-phase iodination with ^{125}I using Iodobeads as the oxidant. Two prewashed Iodobeads were added to 2.5 mCi of [^{125}I]NaI (17.4 Ci/mg of I, NEN/DuPont) in 7 μL of 0.1 N NaOH in a 15-mL polypropylene culture tube, and the volume was adjusted to 4.3 mL with 50 mM $NaPO_4$, pH 8.0. The final NaI concentration was 0.22 μM. APDP was added from a 10 mM stock in acetonitrile to a final concentration of 0.5 mM, and the iodination was allowed to proceed at 0 °C. Samples (1 μL) of the labeling reaction mixture were removed from the Iodobeads at 0, 0.5, 1, 2, 3, and 4 min after the iodination reaction was initiated, thereby terminating the reaction, and spotted onto a silica TLC plate (Analtec, Silica GF 20 × 20 cm). A sample containing 2 nmol in 2 μL of unlabeled APDP was applied as a marker. The plate was developed by ascending chromatography in a solvent consisting of benzene, chloroform, ethyl acetate, and acetic acid (1:1:1:0.1) until the solvent front was 1 cm from the top, dried, and subjected to autoradiography. The unlabeled APDP was visualized under short-wavelength (254-nm) UV light. The analysis of radiolabeled APDP at different times is shown in Figure 1. The R_f of unlabeled APDP was 0.18. The autoradiograph revealed four radioactive spots with R_f's of 0 (origin), 0.19, 0.29, and 0.60. The spot at the origin is unreacted ^{125}I and that with $R_f = 0.6$ is present in the 0 time point (prior to addition of APDP) and is therefore not an iodinated form of APDP. Iodination is nearly complete within 30 s. Reduction of the reagent prior to chromatography results in the disappearance of the 0.19 and 0.29 spots and the appearance of two new spots with lower R_f's (0.10 and 0.16). This indicates that both contained a disulfide bond and that neither of them was produced by cleavage of the disulfide in the other. Spots with R_f values of 0.19 and 0.29 appear to represent iodinated forms of APDP, possibly mono- and diiodinated products. The extent of ^{125}I incorporation did not increase after the initial 30 s of labeling for either iodinated form of APDP. Irradiation of ^{125}I-APDP for 45 s with 254-nm UV light prior to chromatography had no affect on the thin-layer pattern (results not shown).

In order to investigate the relationship between the R_f 0.19 and 0.29 spots, experiments were carried out in which the ^{125}I iodination mixture contained, in addition to Na^{125}I, a 100-

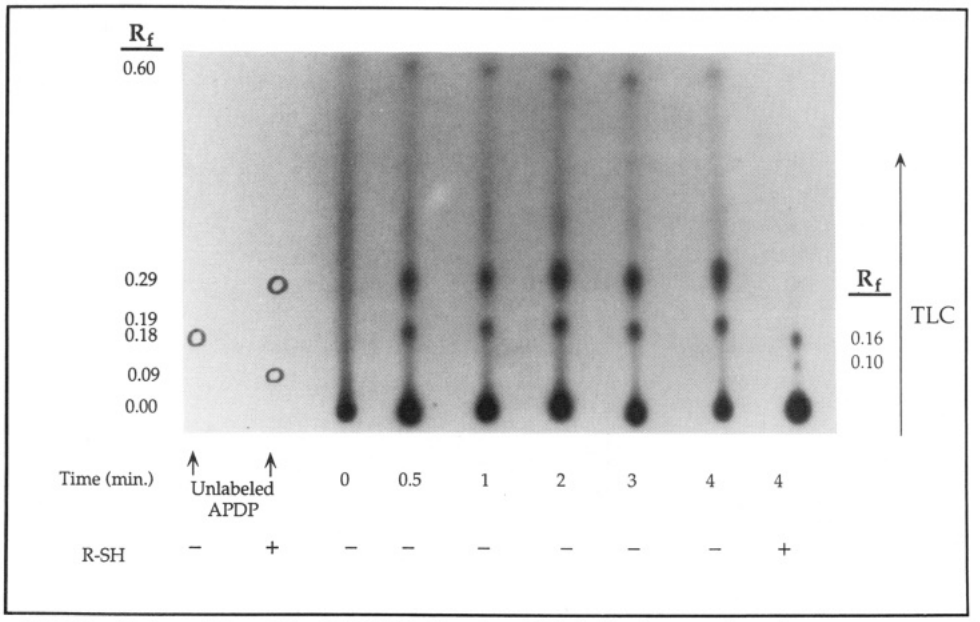

FIGURE 1: Iodination APDP by high specific activity [^{125}I]NaI. Samples of the iodination reaction mixture were removed at the indicated times and analyzed by thin-layer chromatography and autoradiography.

fold molar excess of nonradioactive NaI over APDP. The iodinated APDP was characterized by TLC as above, except that 10 times as much material was applied to the plate to allow detection of the iodinated compounds by both UV visualization and autoradiography. The major UV absorbing material had an R_f of 0.19 and a minor, barely visible, species at R_f 0.29. No UV absorbing material remained at the position of unmodified APDP. As compared to the experiment in Figure 1, there was a shift in the distribution of radioactivity to the 0.19 spot. These results suggest that the component with R_f 0.19 is diiodinated APDP and the component with R_f 0.29 is the monoiodinated APDP. The radiolabeled APDP was used for the modification of L7/L12 without further purification.

Reaction of ^{125}I-APDP with Ribosomal Protein L7/L12Cys89. L7/L12Cys89 (2 mg), determined by Bradford protein assay (Bradford, 1976) against bovine serum albumin as a standard, was completely reduced by incubation for 15 min at 37 °C with 1% (v/v) 2-mercaptoethanol in 2 mL of 50 mM $NaPO_4$, pH 8.0, separated from reducing agent, and stored on ice. The protein concentration was determined to be 0.8 mg/mL. Reduced L7/L12Cys89 (1 mL; 0.8 mg) was mixed with 4.3 mL of 0.5 mM [^{125}I]APDP and incubated for 3 h on ice. The final concentration of the L7/L12Cys89 was 12.4 μM, and that of [^{125}I]APDP was 0.41 mM. Unincorporated [^{125}I]APDP was removed by passage through a Sephadex G-25 column in 10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, and 10 mM $MgCl_2$. In some experiments, L7/L12Cys89 was modified with unlabeled APDP as described above, and the number of free sulfhydryl groups in L7/L12Cys89 and the derivatized protein, APT-L7/L12Cys89,[1] was determined by sulfhydryl titration with [^{14}C]iodoacetamide (Allen, 1989).

The derivatized protein was dialyzed exhaustively against 6% (v/v) acetic acid, lyophilized, and resuspended in 10 mM Tris·HCl, pH 7.4, to give a final concentration of 0.18 mg/mL (15 μM). The absorbance for L7/L12Cys89 and APT-L7/L12Cys89 (15 μM in 10 mM Tris-HCl, pH 7.4) was determined from A_{310} to A_{210}. The specific activity of the labeled protein, determined by scintillation counting, was 1.27 × 10^5 cpm/nmol.

Reconstitution of Ribosomes Containing APT-L7/L12Cys89. Both 70S and 50S core particles lacking L7/L12 were incubated at 37 °C for 15 min in a buffer containing 10 mM Tris·HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 1% (v/v) 2-mercaptoethanol, separated from reducing agent on Sephadex G-25, and stored on ice. ^{125}I-APT-L7/L12Cys89 (50 μg; 4.1 nmol) was added to 1 mg (0.67 nmol) of L7/L12-depleted 50S particles, and the mixture was incubated at 37 °C for 10 min. The ribosomes were collected by centrifugation for 5 h at 58 000 rpm at 4 °C in a Beckman Ti 65 rotor through a 9-mL cushion of 10% (w/v) sucrose in the same buffer, and the ribosome pellet was resuspended in the same buffer, without sucrose, at a concentration of 3 mg/mL. The reconstituted 50S subunits had between 2 and 3 copies of ^{125}I APT-L7/L12Cys89 per particle in different experiments. The same number of copies of L7/L12Cys labeled with [^{14}C]iodoacetamide were bound to the reconstituted 50S subunits after centrifugation. 70S cores were incubated with the same amount of unlabeled APTP and used directly for the assay of activity in protein synthesis without isolation by centrifugation.

Cross-Linking and Identification of Radiolabeled Proteins. Cross-link formation was initiated by irradiation of the samples for 5 min at room temperature with UV light (302 nm from a Transilluminator UV light box, Ultra-Violet Products Inc.) at a distance of 3.5 cm (Traut et al., 1989). Following irradiation the samples were stored on ice. Duplicate 20-μg (6.7-μl) samples of the cross-linked ribosomes were denatured by the addition of SDS[1] to a final concentration of 1% (w/v) with and withiut 0.2 M dithiothreitol and analyzed by SDS-PAGE[1] (Laemmli, 1970) in gels containing 15% (w/v) polyacrylamide. The gels were stained with Coomassie Blue R-250, dried onto filter paper, and subjected to autoradiography. Total cross-linked ribosomal protein was prepared by acetic acid extraction of cross-linked ribosomes (Hardy et al., 1969), and the extracted proteins were dialyzed extensively against 6% (v/v) acetic acid at 4 °C, and lyophilized. Lyophilized proteins were dissolved at 5–10 mg/mL in 20 mM Bistris·acetate, pH 3.7, 8 M urea, and 0.1 M dithithreitol and incubated at room temperature for 4 h. Samples containing 100 μg of protein were analyzed by two-dimensional acid–urea polyacrylamide gel electrophoresis (Knopf et al., 1975) and autoradiography.

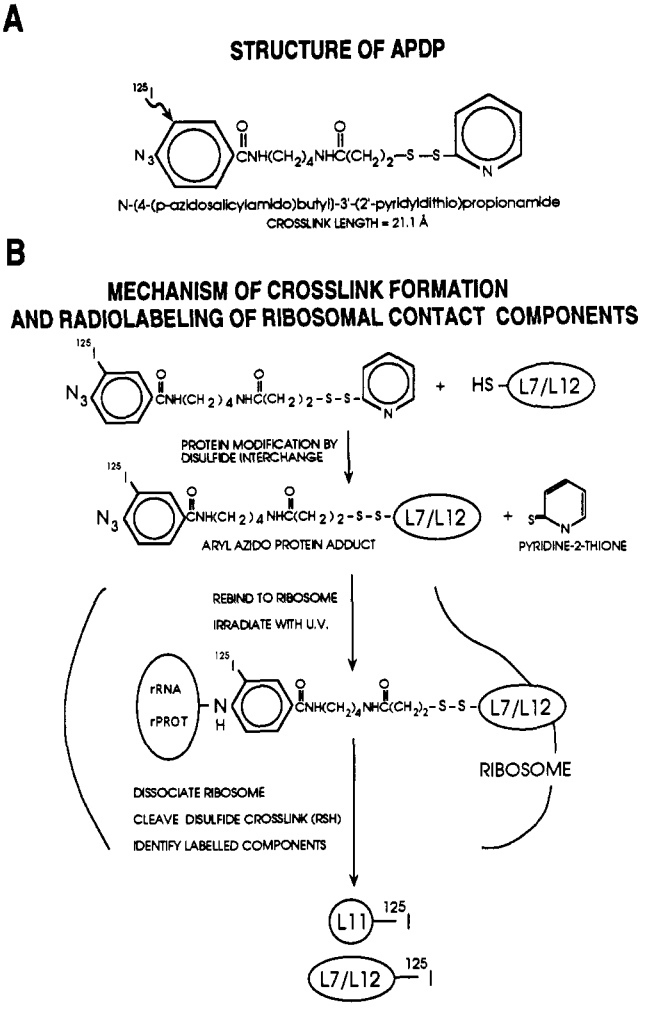

FIGURE 2: The structure of APDP and its mechanism of cross-linking. (A) The structure of the sulfhydryl-specific, heterobifunctional, photoactivatable cross-linking reagent APDP. Radiolabeling with ^{125}I is indicated by an arrow. The maximum length of the cross-bridge is 21.1 Å. (B) Scheme for APDP mediated cross-link formation and radiolabeling of binding site components.

RESULTS

The structure of APDP is shown in Figure 2A. APDP is a heterobifunctional cross-linking reagent that contains two reactive groups, an aryl azide and a dithiopyridyl, separated by a hydrocarbon spacer arm. Figure 2B shows the mechanism of cross-linking. The dithiopyridyl group reacts with protein sulfhydryls by disulfide interchange, displacing pyridine-2-thione and forming a disulfide-linked protein adduct, in this case APT-L7/L12Cys89. The modified protein is reconstituted into 50S ribosomal subunits. These manipulations are performed under normal laboratory fluorescent lighting. APT-L7/L12 acts as a photoactivatable cross-linker due to the presence of an aryl azide moiety. Irradiation with UV light generates a reactive nitrene (Bayley et al., 1977; Ji, 1983) which is capable of covalent reaction with molecules proximal to the C-terminal domain of L7/L12 when it has been specifically reconstituted into 50S subunits, producing disulfide-linked, reversible, covalent cross-links. The reagent can be labeled with ^{125}I prior to protein modification and has the advantage that reductive cleavage of UV-generated cross-links results in the transfer of the ^{125}I label from the originally modified protein to the cross-linked component, thus simplifying identification of components cross-linked to L7/L12.

The absorbance spectrum of APDP in acetonitrile shows maxima at 226, 270, and 310 nm (Figure 3). The maximum

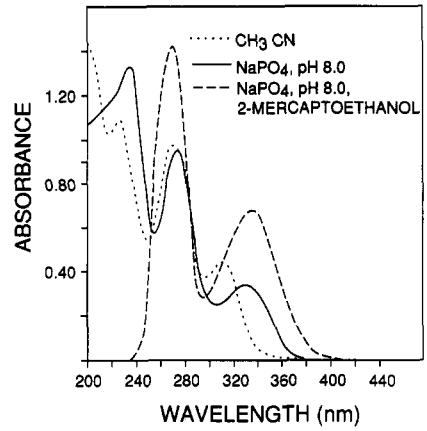

FIGURE 3: Absorbance spectrum of APDP and effect of reduction. APDP dissolved in acetonitrile was diluted into aqueous buffer with or without 2-mercaptoethanol, as indicated. (- - -) 50 μM APDP in CH_3CN; (—) 50 μM APDP in 50 mM $NaPO_4$, pH 8.0; (– –) 50 μM APDP in 50 mM $NaPO_4$, pH 8.0, with 50 mM 2-mercaptoethanol.

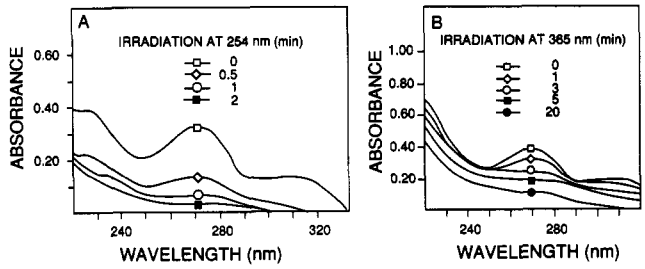

FIGURE 4: Photolysis of APDP by ultraviolet irradiation monitored by absorbance. (A) Irradiation at 254 nm: (□) zero time; (◇) 0.5 min; (○) 1 min; (■) 2 min. (B) Irradiation at 365 nm for the times indicated. (□) zero time; (◇) 1 min; (○) 3 min; (■) 5 min; (●) 20 min.

at 270 nm has been shown in similar reagents to be a characteristic of the azido group and is significantly decreased following photolysis of the reagent (Bayley et al., 1977; Ji, 1979, 1983; Moreland et al., 1982; Peters et al., 1977). The absorbance maxima are shifted to 233, 273, and 330 nm when APDP is diluted from acetonitrile into an aqueous buffer. The average molar extinction coefficient at A_{270} for APDP in acetonitrile calculated from five separate reagent preparations is 1.9×10^4 L mol^{-1} cm^{-1}. Reduction of the reagent results in the appearance of only two absorbance maxima (Figure 3), one near 270 nm and the other at near 340 nm, the latter being close to the published maximum of 343 nm for pyridine-2-thione (Carlsson et al., 1978). The increase in absorbance at 343 nm can be used to monitor the reaction of APDP with protein sulfhydryls as was shown previously for DPDPB (Zecherle et al., 1992). Photolysis of the azido group of APDP by irradiation with UV light causes a sharp decrease in the intensity of absorbance at A_{270} (Figure 4). Photolysis with low-wavelength, 254-nm UV light, (Figure 4A) proceeded more rapidly than with a higher wavelength UV light, 365 nm (Figure 4B). There was no difference in the ultraviolet absorbance spectrum of APDP prepared in the dark or under normal laboratory fluorescent lighting, showing that APDP can be used with normal laboratory lighting and need not be handled in the dark or under red light.

L7/L12Cys89 was derivatized with ^{125}I-APDP without prior removal of unincorporated ^{125}I. Since L7/L12 contains no tyrosine or histidine (Terhorst et al., 1973), it would not be labeled by the excess ^{125}I. Should the method be applied to other proteins that might contain these amino acids, radioiodination at tyrosine and histidine residues would not alter cross-linking results obtained with APDP because the label is reductively transferred to the photo-cross-linked target only

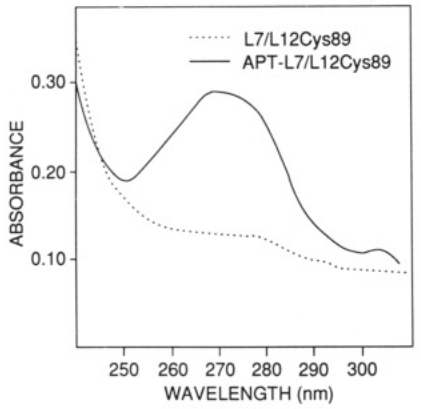

FIGURE 5: Absorbance spectrum of APT-S-S-L7/L12Cys89 formed by reaction with APDP compared to the wild-type or unmodified protein. (- - -) L7/L12Cys89; (—) APT-L7/L12Cys89.

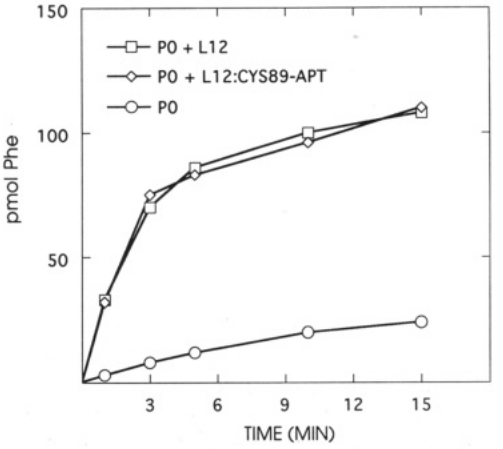

FIGURE 6: Activity of ribosomes reconstituted with L7/L12Cys89 in poly(U)-directed polyphenylalanine synthesis. P0 cores lacking L7/L12 (Hamel et al., 1972) were incubated for 15 min at 37 °C with unmodified or L7/L12Cys89 modified by incubation with APDP in 8-fold excess and diluted into the assay mixture for polyphenylalanine synthesis as described previously (Bartetzko et al., 1988). Reducing agents were absent from the incubation mixture in order to avoid unintended reduction of the disulfide bond in APT-L7/L12. The residual activity of the P0 cores is due to L7/L12 in the supernatant fraction used as the source of tRNA synthetase and elongation factors. The incubation mixtures each contained 20 pmol of P0 cores. The number of phe residues polymerized per ribosome in 15 min for particles reconstituted with wild type or C89 L7/L12 was 10. (□) P0 cores + wild-type L7/L12; (◇) P0 cores + APT-L7/L12Cys89; (○) P0 cores alone.

from the ^{125}I-azidophenylthio moiety of the modified protein (see Figure 2). Modification of L7/L12Cys89 with unlabeled APDP resulted in the complete loss of titratable sulfhydryls from the protein as judged by alkylation with [^{14}C]iodoacetamide (results not shown). The absorbance spectrum for the APT adduct, APT-L7/L12Cys89, shows a maximum at A_{270} not present in the underivatized protein due to the addition of the azido group (Figure 5). The molar extinction coefficient for the derivatized protein at A_{270}, 1.7 × 10^4 L mol^{-1} cm^{-1}, can be used to determine the extent of APDP modification.

The APT-L7/L12 was analyzed by SDS–PAGE[1] and autoradiography following removal of excess ^{125}I and unincorporated ^{125}I-APDP by gel filtration. The L7/L12 became radiolabeled. Reduction of the protein in SDS sample buffer containing 1% (v/v) 2-mercaptoethanol prior to electrophoresis resulted in the complete loss of radioactivity from the protein as judged by SDS–PAGE and autoradiography, consistent with a disulfide linkage between the labeled azido group of APDP and L7/L12 (result not shown).

The 70S ribosomal cores reconstituted with nonradioactive APT-L7/L12 were used to assay for functional activity in polyphenylalanine synthesis. It had previously been shown that the introduction of the Cys89 substitution had no effect on ribosome activity (Zecherle et al., 1992) nor on growth in an allele replacement strain. Figure 6 shows that APT-L7/L12Cys89 is just as active as wild-type L7/L12 in promoting polyphenylalanine synthetic activity when added to P0 ribosomes. It appears that the modifying group does not interfere with functional interactions of the C-terminal domain of L7/L12.

Reconstituted ^{125}I-APT-L7/L12Cys89:50S subunits were used for cross-linking. Photo-cross-linking was initiated by irradiation of the 50S subunits with UV light (302 nm) as described in Materials and Methods, and the ribosomal proteins were analyzed under reducing and nonreducing conditions by one-dimensional SDS–PAGE and autoradiography. Figure 7A shows for reference stained total 50S proteins. The autoradiogram of the gel run under nonreducing conditions (Figure 7B, OX) revealed three major bands that contained radioactivity. The band of lowest molecular weight (apparent M_r 12 200) corresponds to monomeric ^{125}I-APT-L7/L12Cys89. The two other bands have apparent M_r values of 24 000 and 27 000. These two bands disappear following reduction of the protein sample prior to electrophoresis (Figure 7B-RED). This reduced protein shows a broad band not visible with the nonreduced material. The apparent M_r 16 000–18 000 corresponds to that expected for proteins L10, L11, L14, L17, and L20. In the absence of UV irradiation no cross-link formation was observed and irradiation of ribosomes

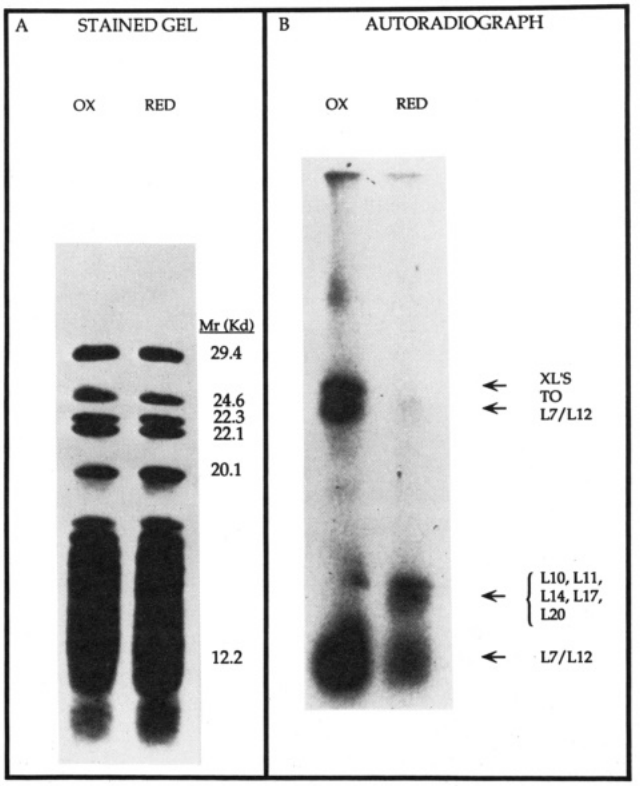

FIGURE 7: Analysis by SDS–PAGE and autoradiography of UV-induced cross-linked proteins from ribosomes containing ^{125}I-APT-S-S-L7/L12Cys89. (A) Stained gel of the total protein extracted from the irradiated, cross-linked 50S ribosomes. (B) Autoradiograph of the same sample.

containing underivatized L7/L12Cys89 did not produce cross-links [results not shown; see also Traut et al. (1989)]. This result suggests that the ^{125}I-APT-L7/L12Cys89 protein adduct was involved in UV-dependent cross-link formation to at least two other ribosomal proteins and that the label is transferred to the cross-linked partner by reductive cleavage of the cross-links.

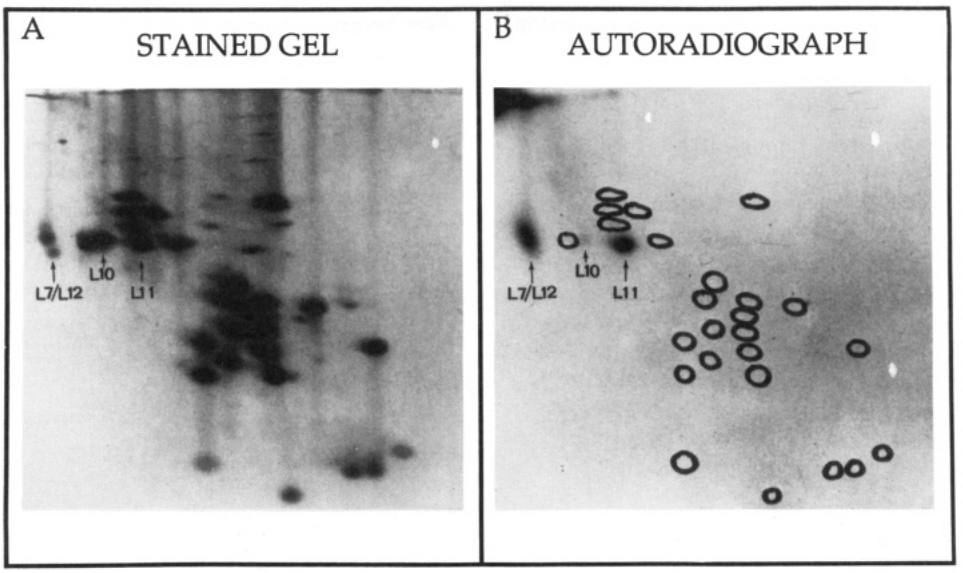

FIGURE 8: Identification of ^{125}I-APDP cross-linked, radiolabeled proteins. The total protein extracted from UV-induced cross-linking of reconstituted 50S subunits containing ^{125}I-APT-S-S-L7/L12Cys89 was reduced by incubation in the presence of 0.1 M DTT in order to transfer the radiolabel to the cross-linked partner prior to two-dimensional acid–urea PAGE. electrophoresis. (A) Stained gel showing all of the 50S proteins and indicating the location of L7, L12, L10, and L11. (B) Autoradiograph.

Two-dimensional acid–urea gel electrophoresis of the fully reduced cross-linked protein in a system that resolves all of the individual proteins was used to determine which of the five candidate proteins implicated by one-dimensional PAGE contained the radiolabel. Figure 8A shows for reference the stained protein spots. The positions of the stained proteins surrounding the radioactive proteins are shown in by circles in Figure 8B. The autoradiograms shown in Figure 8B revealed two major radioactive spots corresponding to L7/L12 and L11, and a faint spot corresponding to L10. The presence of radiolabel in L7/L12 suggests the formation of a intradimer L7/L12–L7/L12 photo-cross-link, since the label that had not reacted, or reacted only with solvent, would have been released from the monomeric L7/L12 seen in Figure 7B-OX. This cross-link is represented by the lower of the two cross-links in Figure 7B-OX. It is also possible that label could have been transferred from Cys89 by photo-cross-linking to a second site within the same monomer. The other major radiolabeled spot corresponds to L11, and the L7/L12–L11 cross-link must be represented by the higher of the two bands in Figure 7B-OX, L7/L12. Since L10 and L11 have nearly the same apparent molecular weights, it is likely that the L7/L12–L10 cross-link is also in the upper oxidized band.

DISCUSSION

Cross-links between L7/L12 and both L10 and L11, as well as other proteins distant from the L7/L12 stalk, using the lysine-specific cross-linking reagent, 2-iminothiolane, have been reported earlier (Traut et al., 1983). The specific lysine residues responsible for cross-linking were not identified. Since there are 14 lysine residues and 2 of them are in the N-terminal domain (residues 4 and 29), and 1 in the hinge (residue 51), it was not even possible to conclude which domain of L7/L12 was involved in cross-link formation. The use of cysteine site-directed mutagenesis to introduce a reactive sulfhydryl at residue 89, a location in the C-terminal domain of L7/L12 predetermined from the crystal structure to be located in a turn between the αB helix and the βB sheet, on the surface of the globular structure has been described (Zecherle et al., 1992). Sulfhydryl-specific cross-linking with ribosomes containing this cysteine-substituted protein necessitates that all the cross-links formed must involve the C-terminal domain of L7/L12. Results with one sulfhydryl-specific cross-linking reagent. DPDPB, were reported earlier (Zecherle et al., 1992), where it was found that Cys89 formed a homodimeric L7/L12–L7/L12 cross-link and a heterodimeric cross-link to Cys 70 of L10. The present work extends those results to identify L11 as a second different protein to which residue 89 in the C-terminal domain can cross-link. Proteins L11 and L10 have been shown to be neighbors in the ribosome by the formation of an L10–L11 cross-link (Expert-Bezancon et al., 1975; Redl et al., 1989; Traut et al., 1980), and by their cooperative binding to the same region of 23S RNA between residues 1052 and 1102 in domain II (Dijk et al., 1979; Egebjerg et al., 1990). The location of both L10 and L11 is also well established by immune electron microscopy to be at the base of the L7/L12 stalk (Nag et al., 1991; Stöffler-Meilicke et al., 1983). The identification of cross-links between Cys89 of L7/L12Cys89 and protein L11 and L10 indicates that the C-terminal domain of at least one dimer of L7/L12 can occupy a site within 21 Å of these proteins at the base of the L7/L12 stalk. The location of the C-terminal domain is thus near that of the N-terminal domain that interacts strongly with L10. The cross-linking results support the model previously proposed on the basis of immune electron microscopy (Olson et al., 1986) that designates two locations for the C-terminal domains of the two L7/L12 dimers, one at the tip of the stalk and a second, less frequently observed, near the N-terminal domain. This implies that L7/L12 can undergo a conformational change, a bending of the extended conformation that exists in the stalk, that brings the C-terminal domain in proximity to the body of the ribosome. This location is consistent with the evidence for the involvement of the C-terminal domain with factor binding and GTP hydrolysis at a site on the body of the 50S subunit near the base of the stalk.

The 50S subunits used in these experiments were reconstituted using L7/L12 that had been purified in the presence of urea. It has been shown that exposure to denaturants makes the binding of one dimer less stable to isolation by high-speed centrifugation (Zantema et al., 1982; this laboratory, unpublished results). The Möller group distinguished a strong and a weak binding site for the two L7/L12 dimers and concluded that the dimer in the strong site was located more on the body of the particle than the stalk (weak site) dimer and that its C-terminal domain was nearer L10. We have reported that the stalk dimer is selectively removed by

incubation of intact 50S particles with a monoclonal antibody against an epitope in the N-terminal domain of L7/L12 (Olson et al., 1986). Preliminary results suggest the dimer that is made more labile to loss by centrifugation by exposure to denaturant is the same as the dimer lost due to the antibody; i.e., the weak site or stalk dimer.[2] It has not been established that the population of ribosomes used in these cross-linking experiments consists of particles with all the L7/L12 dimers in the same location, in the nonstalk or bent conformation, but it seems clear that the L11 and L10 cross-links arise from the dimer in the strong binding site on the body. Despite the weaker binding of one on the dimers with respect to centrifugation, it is clear that the same preparation of L7/L12Cys89 fully modified with APTP restored activity in protein synthesis when the particles were assayed directly without isolation by centrifugation. This confirms the results of Zantema et al. (1982).

The C-terminal domain of L7/12 has been shown to be important in the factor-related steps of protein biosynthesis, including the binding of elongation factors EF-Tu and EF-G, and the factor-dependent hydrolysis of GTP (Koteliansky et al., 1978; Sommer et al., 1985; Van Agthoven et al., 1975). Since the locations for EF-Tu and EF-G binding on the ribosome are at the base of the stalk (Girshovich et al., 1981; Maassen et al., 1978, 1974; Rychlik et al., 1983; San Jose et al., 1976), we propose that the bent conformation of L7/L12 is involved in factor binding to the body of the 50S subunit. The role of the stalk C-terminal domain that is distant from the site of this functional interaction, as well as the presence of two dimers, and the dynamics of the two conformations and locations in relation to protein synthesis remain to be explained.

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