Location and domain structure of *Escherichia coli* ribosomal protein L7/L12: site specific cysteine cross-linking and attachment of fluorescent probes

Robert R. Traut, Debendranath Dey, Dmitry E. Bochkariov, Andrew V. Oleinikov, George G. Jokhadze, Brian Hamman, and David Jameson

Abstract: Five different variants of L7/L12 containing single cysteine substitutions, two in the N-terminal (NTD) and three in the C-terminal domain (CTD), were produced, modified with [^{125}I]*N*-[4-(*p*-azido-salicylamido)butyl]-3-(2′-pyridyldithio) propionamide ([^{125}I]APDP), a sulfhydryl-specific, heterobifunctional, cleavable photo-cross-linking reagent, and reconstituted into ribosomes. These were irradiated, the total proteins were extracted and reductively cleaved, and the cross-linked proteins were identified. The effect of zero-length disulfide cross-linking on binding and activity was also determined. The same sites in L7/L12 were used to attach a rhodamine dye. The formation of ground-state rhodamine dimers caused the appearance of a new absorption band at 518 nm that was used to estimate the extent of interaction of the probes in the free protein and in complexes with L10. The three sites in the CTD, but not the N-terminal sites, cross-linked to L2 and L5 and to 30S proteins S2, S3, S7, S14, and S18 in a manner influenced by elongation factors. Binding to the ribosome and, therefore, function were blocked by zero-length cross-linking within the NTD, but not the CTD. Binding also disrupted rhodamine dimers in the NTD. No rhodamine dimers formed in the CTD.

Key words: ribosomes; L7/L12; cross-links; rhodamine dimers.

Résumé : Cinq protéines L7/L12 mutantes différentes dans lesquelles il y a substitution d'une seule cystéine, dans le domaine N-terminal de deux d'entre elles et dans le domaine C-terminal des trois autres, ont été synthétisées et modifiées en utilisant le [^{125}I]-*N*-4-(*p*-azidosalicylamido)-butyl-3-(2′-pyridyldithio) propionamide (APDP), un réactif de photoréticulation hétérobifonctionnel, hydrolysable et spécifique des groupes thiol. Puis, elles ont été incorporées dans des ribosomes reconstitués. Ces ribosomes ont été irradiés, toutes les protéines ont été extraites et fragmentées par réduction, puis les protéines réticulées ont été identifiées. L'effet de simples ponts disulfure sur la liaison et l'activité a aussi été déterminé. La rhodamine a été fixée aux mêmes sites des protéines L7/L12. La formation de dimères de rhodamine entraîne l'apparition spontanée d'un nouveau pic d'absorption à 518 nm qui a été utilisé pour estimer l'ampleur de l'interaction entre les marqueurs dans chaque protéine à l'état libre ou formant un complexe avec la protéine L10. Les trois résidus cystéine à l'extrémité C, mais non ceux à l'extrémité N, forment des ponts avec les protéines L2 et L5, ainsi qu'avec les protéines S2, S3, S7, S14 et S18 de la sous-unité 30S. Ce processus est affecté par les facteurs d'élongation. La liaison au ribosome, et ainsi leur fonction, est inhibée lorsque de simples ponts disulfure intramoléculaires sont présents dans le domaine N-terminal, mais non dans le domaine C-terminal. La liaison est également affectée par les dimères de rhodamine à l'extrémité N. Aucun dimère de rhodamine ne se forme à l'extrémité C.

Mots clés : ribosomes, protéine L7/L12, réticulation, dimère de rhodamine.

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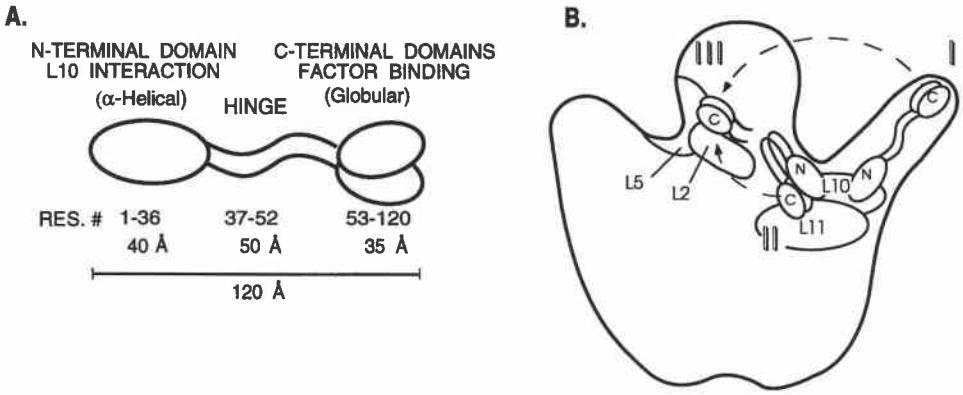
Abbreviations: NTD, N-terminal domain; CTD, C-terminal domain; APDP, *N*-[4-(*p*-azidosalicylamido)butyl]-3-(2′-pyridyldithio) propionamide; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate, P0, protein deficient ribosomal core particles lacking completely and specifically L7/L12.

R.R. Traut,[1] **D. Dey, D.E. Bochkariov, A.V. Oleinikov, and G.G. Jokhadze.** Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616, U.S.A.
B. Hamman and D. Jameson. Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, HI 96822, U.S.A.

[1] Author to whom all correspondence should be addressed.

Fig. 1. (A) The domain structure of L7/L12 and (B) the alternative locations (I, II, III) and orientations on the 50S ribosomal subunit.



Introduction

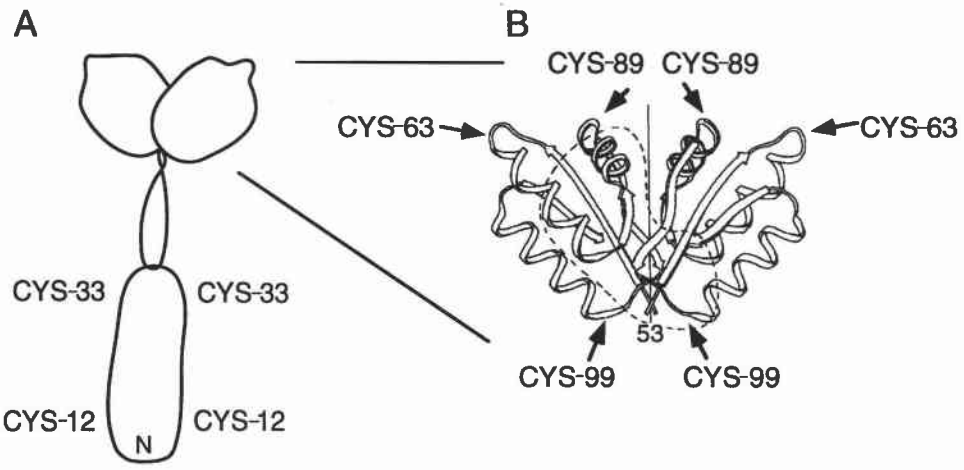
Ribosomal protein L7/L12 of *Escherichia coli* is the most extensively investigated representative of the small, four-copy, dimeric acidic proteins found in large ribosomal subunits of all organisms. L7 is a post-translationally N-terminal acetylated form of the L12 gene product from which it is functionally indistinguishable. In eubacteria, eukaryotes, and archaea, the acidic 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 (Liljas 1982; Uchiumi et al. 1987; Casiano et al. 1990). One or both of the L7/L12 dimers forms a conspicuous morphological feature on the ribosome known in *E. coli* as the L7/L12 stalk (Strycharz et al. 1978). The proteins can be simply and selectively removed from and reconstituted into the ribosome (Hamel et al. 1972). In both eubacteria and eukaryotes, the proteins are required for the binding of translational factors. The association between these ribosomal proteins and factors to produce GTP hydrolysis derived energy for template-guided movement of the ribosome is of interest with respect to the ribosome and also as an example of a mechanochemical system (Kischa et al. 1971), or molecular motor, and as an effector G protein system. This is a major example of ribosome function in which specific proteins play a defined and perhaps dominant role. However, major questions concerning why dimers are the functional element and why there are two dimers remain unanswered. The general background underlying the work presented here has been described previously (Traut et al. 1993).

The domain structure of protein L7/L12 of *E. coli* with approximate extended dimensions is shown in Fig. 1A. The maximally extended length of the molecule (125 Å (1 Å = 0.1 nm)) is slightly less than half the distance across the surface of the 50S subunit (Lake 1976). It is composed of two distinct organized structural domains linked by a flexible hinge (Leijonmarck et al. 1981; Liljas and Gudkov 1987). The elongated, helical N-terminal domain (NTD), residues 1–36, is responsible for the strong dimer interaction (Gudkov and Behlke 1978) through a likely coiled-coil interaction of the two α-helices (Möller and Maassen 1986; Tsurugi and Mitsui 1991). The globular C-terminal domain (CTD), residues 53–120, has been implicated in factor binding in that 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; 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). It was proposed from the crystallographic structure that two CTDs together formed a conserved surface of possible functional importance (Leijonmarck and Liljas 1987). Flexibility of L7/L12 has been demonstrated both by proton NMR (Gudkov et al. 1982; Cowgill et al. 1984) and by electron microscopy (Verschoor et al. 1986). Determining more precisely the ribosomal location of the functionally important C-terminal domains has continued to be a goal of work in this laboratory. Present information concerning the location of the C-terminal domain is summarized in Fig. 1B. It represents three locations for the C-terminal domain, numbered I, II, and III, and two conformations for the L7/L12 dimer, bent (II) and extended (I, the stalk, and III).

It is well established from electron microscopy that L7/L12 comprises the stalk (I) observed on large ribosomal subunits. The location of the CTD at the tip of the stalk was inferred from the demonstration that the N-terminal domain is responsible for binding to L10 (Agthoven et al. 1975; Koteliansky et al. 1978), and it is shown directly by immunoelectron microscopy with monoclonal antibodies (Olson et al. 1986), which suggested that both dimers were present in the stalk (Tokimatsu et al. 1981); however, it was also shown that one dimer per particle was sufficient to form a visible stalk (Möller et al. 1983). The clearly established location of EF–G on the body of the ribosome near the base of the stalk (site II) (Girshovich et al. 1981) appears to be incompatible with the static presence of the functionally required C-terminal domains of both dimers at the end of the stalk.

Direct evidence for the location of the C-terminal domain at site II first came from immunoelectron microscopy with the monoclonal antibody mentioned above, which showed a secondary antibody binding site at the periphery of stalkless subunits in an area from which the stalk might be expected to project (Olson et al. 1986), i.e., near the site of EF–G binding. The Leiden group (Möller and Maassen 1986) provided early evidence for the location of one L7/L12 dimer on the body of the 50S particle in an extended conformation directed toward the central protuberance. Additional evidence that a CTD can occupy a location on the body of the ribosome 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 (Zecherle et al. 1992*b*; Makarov et al. 1993). A different het-

Fig. 2. (A) Sites of cysteine site directed mutagenesis in the domain structure of L7/L12 and (B) their location in the X-ray crystallographic structure. The amino acids substituted by cysteine were Ala-12, Ser-33, Ala-63, Ser-89, and Ser-99.



erobifunctional reagent [^{125}I]APDP showed a cross-link between Cys-89 in the CTD and L11, also near the EF–G binding site near the base of the stalk (Zecherle et al. 1992*a*).

Previous studies on the identification of protein–protein cross-links involving L7/L12, using derivatization of ribosomal lysine residues with 2-iminothiolane and oxidative disulfide cross-linking between introduced and (or) endogenous SH groups, showed that L7/L12 was able to form cross-links with proteins near the base of the stalk and also with proteins near the 5S RNA (L5) and peptidyl transferase domains (L2), regions distant both from the stalk and the EF–G binding site (Traut et al. 1983, 1986). A cross-link between L7/L12 and L5 has also been reported using a different lysine-specific cross-linking and detection method (Redl et al. 1989). As indicated in Fig. 1, the fully extended length of L7/L12 is estimated to be up to 125 Å. This length is sufficient to permit either the stalk or the bent CTD to reach site III.

We have attempted to obtain a more precise correlation of the locations of the C-terminal domains and the functional state of the ribosome. Any detailed understanding of molecular mechanisms needs to account for the existence of two dimers, transitions between different locations, and conformations. Possible models and dynamics have been discussed by Möller (Möller and Maassen 1986; Möller 1990) and by Liljas and Gudkov (1987). Implicit in these transitions is the flexibility of L7/L12. Occupancy of site II with the dimer in the bent conformation clearly requires flexibility and is presumed to be conferred by the hinge region. A transition between sites I and III would require a different kind of motion, a "ball-and-socket" binding of L7/L12 to L10 that allows the extended dimer to swivel between the stalk and inward locations. Alternatively, the transition between sites II and III may be the relevant one.

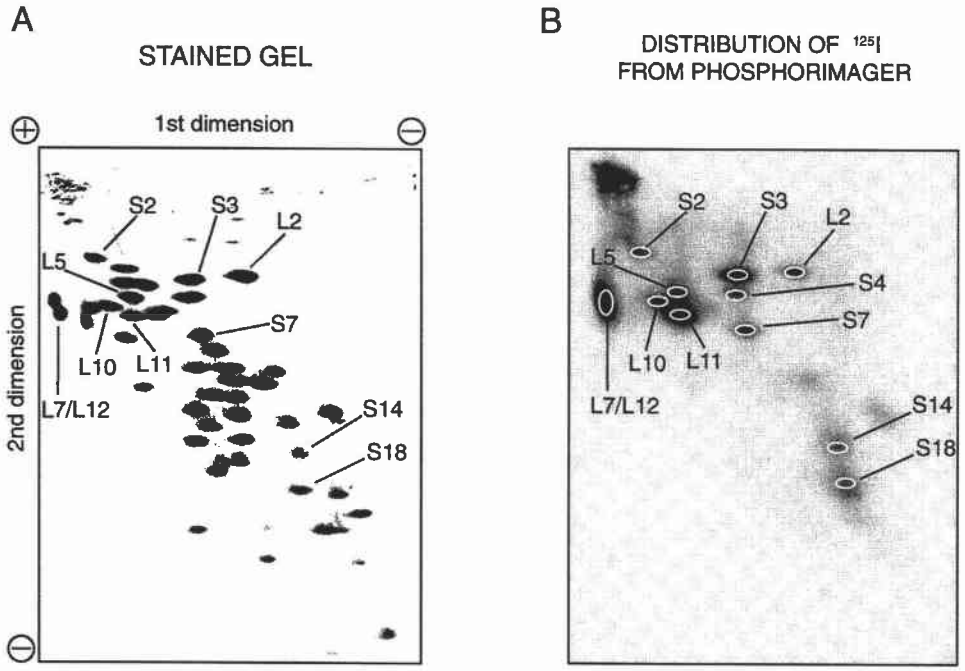
Results

Cysteine substitution by site-directed mutagenesis and sulfhydryl cross-linking as a probe of the location and dynamics of L7/L12

The primary structure of L7/L12 was available and showed the absence of cysteine residues in the wild-type protein from *E. coli* (Terhorst et al. 1973). The locations of the five single cysteine substitutions made in L7/L12 in the accepted model, showing the nonstaggered, parallel arrangement of the dimer (Liljas 1982), are shown in Fig. 2A. The protein variants were overexpressed and purified by methods described previously (Oleinikov et al. 1993*a*, 1993*b*). The location of residues 63, 89, and 99 are represented in the model based on the high resolution crystallographic structure of the CTD as shown in Fig. 2B. Residue 89 is located in the turn between the αB helix and the βB sheet, residue 63 is located in the turn between the βA sheet and the αA helix, and residue 99 is in the turn between the βB sheet and the αC helix (Leijonmarck and Liljas 1987). The three sites are on exposed surfaces facing in different directions. Residue 99 is near residue 53, the junction of the CTD with the hinge, residue 33 is in the N-terminal domain near the junction with the hinge, and residue 12 is near the N-terminus of the molecule. Cysteine substitution had no effect on the activity of reconstituted particles, not even when iodoacetamide or more bulky moieties were attached at these sites (Zecherle et al. 1992*a*).

Each of the purified L7/L12 variants was modified at the single cysteine sulfhydryl with the radioiodinated heterobifunctional photo-cross-linking reagent [^{125}I]APDP. As described previously (Zecherle et al. 1992*a*), the radioactive, photoactivatable azido moiety is attached to L7/L12 through formation of a cleavable disulfide bond. Specific activity of [^{125}I]APDP was adjusted to 10 Ci/mmol (1 Ci = 37 GBq), and the extent of protein modification was routinely 90–95%. The modified proteins, purified from unreacted reagent, were reconstituted into 70S ribosomal core particles completely lacking L7/L12 (denoted by P0 (Hamel et al. 1972)). The reconstituted ribosomes were isolated by centrifugation through a sucrose cushion, resuspended, and irradiated. The total proteins were extracted and reduced to cleave monomeric target proteins from their disulfide attachment to L7/L12 with transfer of the label to the target. The monomeric proteins were separated by two-dimensional PAGE (Kenny et al. 1979), stained, and analyzed for ^{125}I distribution using a Bio-Rad phosphorimager. A typical result for the Cys-63 variant is shown in Fig. 3. The stained gel is shown in Fig. 3A and those proteins that became radiolabeled are indicated. The gel system separates most 70S ribosomal proteins with good resolu-

Fig. 3. The separation and analysis of reduced, monomeric ^{125}I-labeled proteins by two-dimensional PAGE. (A) The stained pattern showing all of the 70S proteins. (B) The phosphorimager analysis of the distribution of radiolabel in the same gel. An experiment with the labeled protein [^{125}I]APDP–Cys63 is reproduced in this example.



tion, and this is the case for the labeled proteins. The distribution of labeled proteins generated by the phosphorimager is shown in Fig. 3B. Proteins L7/L12, L10, and L11 are major labeled proteins. Proteins L2 and L5 are labeled to a much less, but clearly significant, extent. The 30S ribosomal proteins S2, S3, S7, S14, and S18 are labeled. Protein S4 is visible in this gel but is generally found only at a very low level and only with Cys-63. Only a very low level of labeling occurred in the absence of irradiation because of the failure to completely exclude stray light. 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 only 30S subunits, a low level of only S2 labeling occurred in addition to homolabeling. All variants were tested for transfer of radiolabel to RNA, and none was detected.

The phosphorimager was programmed to quantify the amount of radioactivity present in each protein spot relative to the total detected on the gel. The results for Cys-12 and Cys-63 are shown in Fig. 4. Similar graphs were obtained for all five variants. The height of the bars provides a linear depiction of the relative distribution of the extent of labeling among the total proteins. There is clearly a background level, and we have drawn a horizontal line across the graph to denote a threshold above which we consider labeling to be significant. The proteins listed qualitatively above appear as bars of different heights above the threshold level. To summarize the labeling of 10 different proteins from five different sites, we have arbitrarily divided the bar graphs into four levels above the threshold and denote the extent of labeling by the number of + symbols from + (weakest labeling) to ++++ (strongest labeling). These results are given in Table 1.

Cross-linking to other proteins from the NTD sites was limited to proteins L10 and L11, with L10 the strongest. The labeling of L10 from both Cys-12 and Cys-33 greatly exceeded L10 labeling from any of the CTD sites. 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 NTD variants was much greater than for the CTD variants, with Cys-63 distinctly lower than Cys-89 and Cys-99. Ribosome binding had no effect on the CTD sites but greatly lowered the Cys-12 homolabeling and reduced Cys-33 homolabeling.

Cross-linking from the three CTD sites was strongest to L11, a result reported previously for Cys-89 (Zecherle et al. 1992*a*). Cross-linking to L10 was lower than to L11 but was also strong. Homolabeling of L7/L12 was approximately the same as labeling of L10 and L11. Weaker, but significant, labeling of L2 and L5, cross-links found previously with 2-iminothiolane (Traut et al. 1983), was detected from all three CTD sites. In general, Cys-63 gave the highest yields of cross-linking to all of the other 50S proteins, and Cys-99 cross-linked weakly to all proteins except L11. The CTD sites cross-linked to certain 30S proteins, more strongly to S3 but significantly to S2, S7, S14, and S18. The different labeling patterns for the different Cys residues again illustrates the specificity of the photochemical reaction and the absence of labeling by chemical disulfide interchange.

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:GMPPNP, and the EF–Tu:GMPPNP:Phe t-RNA ternary complex. Neither of the EF–G complexes gave any consistent, major alterations in the distribution of cross-links. The results with the EF–Tu ternary complex are shown in Fig.

Fig. 4. Quantitative analysis of the distribution of radiolabel obtained with the phosphorimager. The instrument was programmed to quantify the center part of equal area for each labeled spot to minimize overlap (the white ovals in Fig. 3B). The bar heights represent linearly the relative amounts of radioactivity in each protein. The indices and + symbols on the right ordinate designate the levels of radiolabel summarized in Table 1.

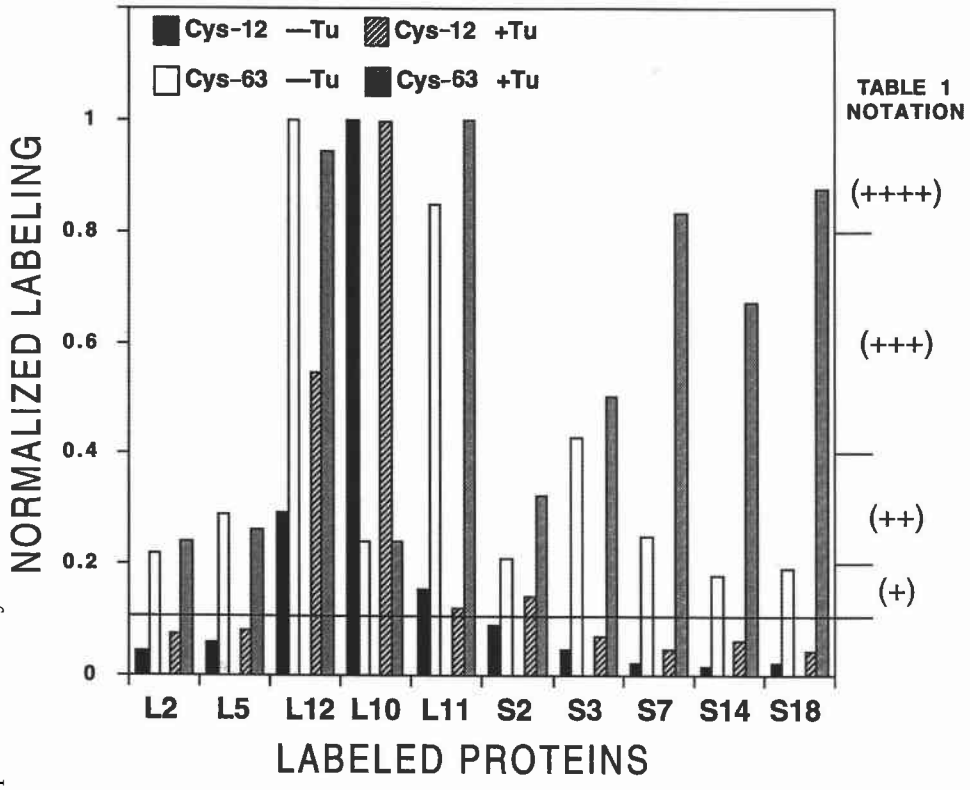


Table 1. Summary of proteins labeled by [^{125}I]APDP attached to different cysteines in L7/L12.

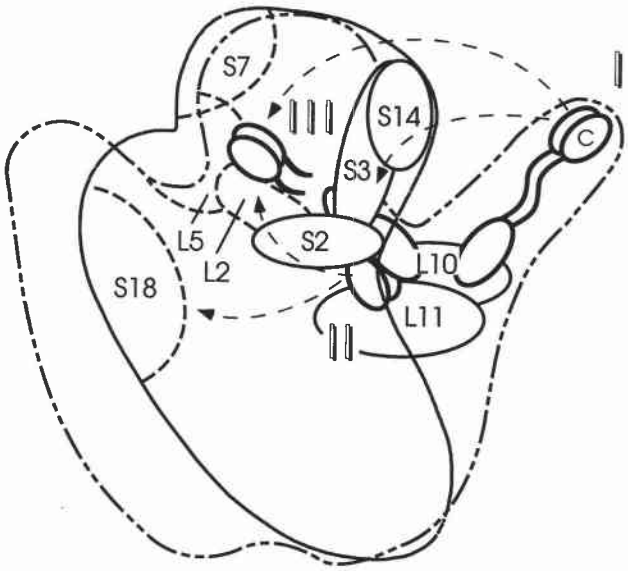
	70S					70S·polyU + EF-Tu·GppNp·Phe-tRNA				
	NTD		CTD			NTD		CTD		
	12	33	63	89	99	12	33	63	89	99
L2	–	–	++	+	+	–	–	++	+	–
L5	–	+	++	++	++	–	+	++	+	+
L7/L12	++	++++	++++	++++	++++	+++	++++	++++	++++	++++
L10	++++	++++	++	++	+	++++	+++	++	+	+
L11	+	+++	++++	++++	++++	+	++	++++	++	+++
S2	–	+	++	++	+	+	++	++	++	–
S3	–	–	+++	+++	+++	–	+	+++	++	++
S7	–	–	++	+	+	–	–	++++	++	+++
S14	–	–	+	+	+	–	–	+++	++	++
S18	–	–	+	–	++	–	–	++++	+	++

Note: The + symbols refer to the relative levels of labeling designated in Fig. 4: +, weakest; ++++, strongest.

4, which compares the extent of labeling of the 10 major proteins in the absence and presence of the factor. The results for all five variants are summarized in Table 1. There is a consistent increase in the labeling of small subunit proteins S7, S14, and S18 in the presence of the factor.

The location on the ribosome of the proteins cross-linked from the various sites in L7/L12 is shown in Fig. 5. Evidence for the location of 50S cross-linked proteins L10, L11, L2, and L5 has been presented previously (Traut et al. 1993). The locations of the 30S proteins are taken from the immunoelectron microscopy studies of Lake (Scheinman et al. 1992) and Stöffler (Stöffler-Meilicke and Stöffler 1990). The 30S subunit is shown with the platform on the left, opposite the L7/L12 stalk. The broken outlines designate proteins on the subunit inter-

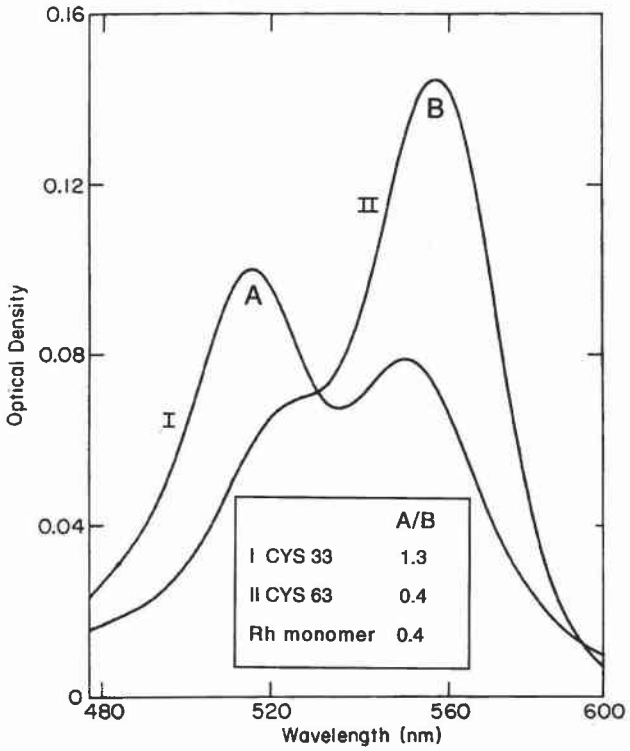
Fig. 5. A model of the 50S and 30S ribosomal subunits in the 70S ribosome, showing the major cross-linked proteins. Broken lines indicate proteins on the subunit interface surface of the subunits. Arrows represent motion or conformations of L7/L12 likely to be involved in the various cross-links.



face surfaces of the subunits. The arrows suggest movement or conformation, of L7/L12 consistent with cross-linking to the target proteins. The strong labeling of L10, but not of the distant proteins L2 and L5 or 30S proteins, from the NTD sites is consistent with the anchoring of the NTD through L10. The labeling of the distant proteins L2 and L5 from the CTD sites, but not from the NTD sites, is consistent with both the length and flexibility of L7/L12. The cross-linking reagent adds 21 Å to the extended length of L7/L12 and either the stalk dimer or the inward, bent dimer could make these contacts. The result extends the earlier cross-linking study with the lysine specific reagent 2-iminothiolane (Traut et al. 1983) and defines the CTD as the relevant domain. In that study, L7/L12–L5 cross-link was identified in 50S but not in 70S ribosomes, and it was suggested that competition for L5 lysines by 30S interface proteins could account for this finding. The present result with 70S ribosomes implies that 30S binding does not block access across the interface cavity. Inspection of the X-ray crystallographic structure of the CTD shows that, of the sites studied, Cys-63 is maximally exposed and distant from the N-terminus, and its formation of cross-links in highest yield is consistent with this. The location of Cys-99 on the region of the CTD nearest the N-terminus is consistent with its much lower yield of cross-linking.

The cross-linking of 30S proteins was an unexpected result since none had been detected with 2-iminothiolane. Again, competition for lysine cross-linking sites may explain the differing results. Three of the cross-linked 30S proteins, S2, S3, and S14, are clustered on the exterior of the head of the 30S subunit on the same side of the 70S particle as the L7/L12 stalk. The location of this group of proteins coincides nearly perfectly with that determined by immunoelectron microscopy for EF–Tu (Girshovich et al. 1986; Langer and Lake 1986). Movement of the flexible stalk dimer could bring the CTD near these proteins. The results suggest the participation of the

Fig. 6. The absorption spectra of L7/L12:rhodamine conjugates for L7/L12 Cys-33 (spectrum I) and Cys-63 (spectrum II) in solution. Peak A, 518 nm; peak B, 555 nm. The spectrum for Cys-12 was virtually identical with I, and those for Cys-89 and Cys-99 were virtually identical with II. The spectrum for monomeric rhodamine is not shown. The insert box shows the ratio of 555–518 nm for Cys-33, Cys-63, and the rhodamine monomer.



CTD of L7/L12 in EF–Tu binding at the R site and thus imply a role for L7/L12 in factor binding at a site other than the EF–G site at the base of the stalk. The two elongation factors compete for binding at this site, and EF–Tu has been identified in a cross-linked complex containing L7/L12 and L10 (B. Nag, R.R. Traut, and A.E. Johnson, unpublished results). Experiments are in progress to analyze the labeling of factors from the L7/L12 Cys sites. The stimulation of labeling of 30S proteins by EF–Tu is consistent with a conformational change that brings L7/L12 in closer proximity to sites on the small subunit. Labeling of S7 and S18 is stimulated the most by EF–Tu. The proteins are well separated from each other and are distant from the S2–S3–S14 cluster. Protein S7 is located near L5 and L2, and the orientation of L7/L12 that led to their labeling can also account for that of S7. The labeling of S18 is not readily explained in the static model depicted. The significant increase in S18 labeling caused by EF–Tu suggests a conformational change that might rotate the 30S subunit to bring the S18 site nearer the interface cavity, where contact with the inner dimer could take place.

In summary, the results of cross-linking by attaching the sulfhydryl-specific heterobifunctional reagent APDP to cysteine residues in the CTD of L7/L12 confirm three possible locations for these globular domains, or three orientations of the molecule. Cross-linking of L11 and L10 confirms the bent conformation with the CTD at the base of the stalk (site II).

Fig. 7. A model summarizing (A) the locations at which rhodamine dimers do (NTD) or do not (CTD) form and (B) the effect of binding to L10 on the NTD rhodamine dimers.

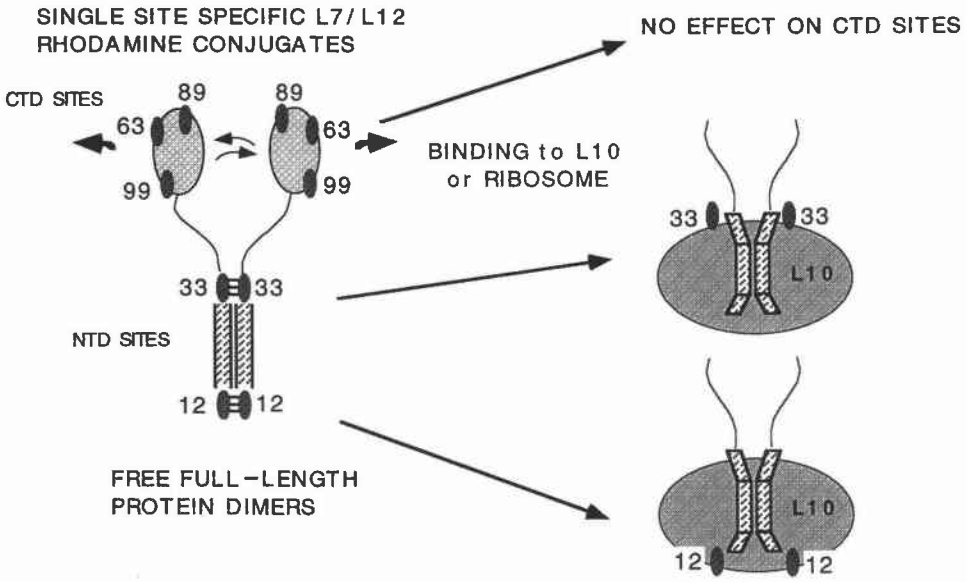


Table 2. Rhodamine dimer formation at cysteine sites in the N-terminal domain of L7/L12.

	Ratio 518/555
Evidence for subunit exchange in L7/L12 dimers:	
Wild type/Cys-33–rhodamine, Molar ratio, 0	1.3
Molar ratio, 1	0.8
Molar ratio, 10	0.4
Effect of binding to L10 or ribosomes:	
Cys-33–rhodamine	1.3
+ L10 (1:1)	0.5
+ 70S PO (1:1)	0.5

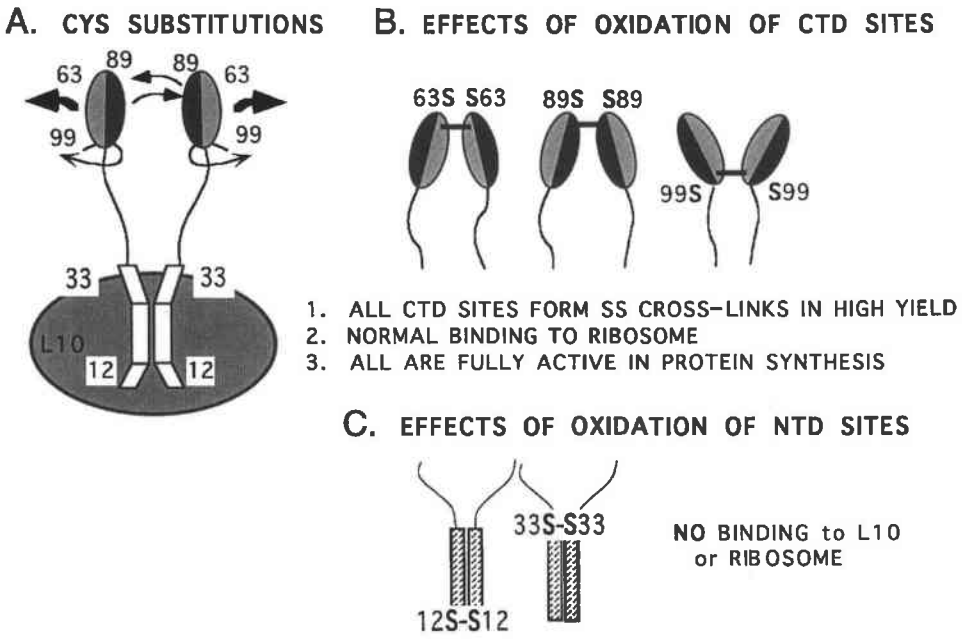
Cross-linking of L2 and L5 confirms an extended orientation across the body of the 50S subunit (site III). Cross-linking of S2, S3, and S14 shows one possible location of the stalk, stretching across the subunit interface to reach the EF–Tu binding site on the small subunit. These three locations appear to represent specific, stable conformations. The probe does not sweep out a swath of labeled proteins around a radius from its anchoring site or from the junction of the hinge and anchoring NTD. Site II is clearly of functional significance because it coincides with the well established EF–G binding location. Our results also suggest a functional role for site I, the extended stalk. It may make an initial contact with EF–Tu in the 30S site and be involved in its movement to site II, at which EF–Tu and EF–G have overlapping sites and compete (see Liljas 1982).

Attachment of spectroscopic probes to L7/L12 cysteine residues and the use of tetramethylrhodamine dimer formation to study the conformation and dynamics of L7/L12 dimers

Cysteine site directed mutagenesis opens up the possibility of using the selected cysteine residues as points for the attachment of sulfhydryl-specific spectroscopic probes (Falke and Koshland 1987). Such studies can reveal new aspects concerning the local environments of each site and facilitate determinations of distance-dependent energy transfer efficiency between two sites in L7/L12 or single sites and other locations on the ribosome. Initial studies have utilized a sulfhydryl-specific fluorescent rhodamine derivative (tetramethylrhodamine iodoacetamide). Ground-state tetramethylrhodamine acetamide dimers can form between the two subunits of L7/L12 depending on the location of the probe. The formation of tetramethylrhodamine dimers causes the appearance of a new absorption band at 518 nm. The formation of the new 518 nm absorbance peak characteristic of the rhodamine–rhodamine interaction was dependent on the domain in which the probe was located. As shown in Fig. 6, the probe located at Cys-33 in the NTD displayed the 518 nm band, whereas the probe at Cys-63 in the CTD did not. The spectrum for the probe at Cys-12 was virtually identical with Cys-33, and those for the derivatives at Cys-89 and Cys-99 were virtually identical with Cys-63. The ratio of the intensities of the new 518 nm band to the preexisting 555 nm band served as a convenient way of quantifying the spectroscopic data. This is indicated in the insert box in the figure. Intersubunit tetramethylrhodamine dimers, as indicated by peak B, disappear on addition of guanidine hydrochloride and reform on removal of the denaturant. The absence of rhodamine dimers in the CTD sites is consistent with the absence of energy transfer between probes attached to these sites (B. Hamman and D. Jameson, unpublished results).

The tetramethylrhodamine dimers in L7/L12:Cys-12 or Cys-33 disappear rapidly on addition of excess unlabeled wild-type L7/L12, as indicated by the fall in the 518–555 ratio (Table 2). It appears that singly labeled L7/L12 dimers are formed by exchange with wild-type dimers. Exchange has also been noted when the 1–52 NTD fragment was mixed with full-length dimers and cross-linked (A. Oleinikov and R.R. Traut, unpublished results). Cross-linked dimers of molecular weight between the two homodimers were detected and quantified on SDS–PAGE.

Fig. 8. A model showing (A) the sites of zero-length disulfide cross-linking in the five L7/L12 cysteine variants and the formation and effects of the cross-links (B) in the C-terminal domain and (C) in the N-terminal domain. Bold arrows (A) represent movements of the C-terminal domain to different sites (I, II, III) as shown in Fig. 5; light arrows represent relative movements and rotation of the C-terminal domain within the quaternary structure of L7/L12.



The binding of L7/L12 : tetramethylrhodamine Cys-33 or Cys-12 either to L7/L12-depleted ribosomal core particles or to ribosomal protein L10 alone led to the disappearance of the 518 nm absorption band, i.e., to the disruption of rhodamine dimers (Table 2). This result implies different conformations for both ends of the NTD of L7/L12 dependent on whether L7/L12 is free or bound to L10 or to the ribosome. The results are highly consistent with the failure of the covalent disulfide-linked NTD dimers to bind to the ribosome and the restoration of binding by cleaving the covalent bond (Oleinikov et al. 1993*a*).

These observations are summarized in schematic form in Fig. 7. The locations of all five rhodamine conjugates are summarized in the left-hand image of Fig. 7, which indicates the formation of rhodamine dimers in the two N-terminal locations but not in either of the three C-terminal locations. The right-hand images depict the absence of rhodamine dimers with ribosome-bound Cys-12 and Cys-33 and the separation of these regions of the polypeptide chains induced by binding.

Direct zero-length cross-linking of Cys variants and its effect on ribosome binding and activity

Previous publications have described the behavior of Cys-63, Cys-89, and Cys-33 following oxidation with Cu^{2+}(phenanthroline)$_3$ to promote disulfide bond formation (Oleinikov et al. 1993*a*). Disulfide bond formation took place to completion in all three cases. The two dimers cross-linked at the CTD sites fully restored activity to P0 cores. It was shown that the proteins retained the disulfide bonds after the assay for protein synthesis. Cross-linking at residue 33 in the NTD abolished its capacity to bind to the ribosome. It became active after reduction of the disulfide bond. This approach has been extended to the two additional sites, Cys 99 and Cys-12, located as described earlier and indicated in Fig. 2. These two variants were also oxidized to completion, confirming the conclusion that the CTD is flexible and indicating that residues 12 near the N-terminus either face each other in a putative coiled-coil structure or have significant freedom of rotation. Both variants were added to P0 core particles that lacked L7/L12 and were tested for activity in a system lacking the usual reducing agents as described previously (Oleinikov et al. 1993*b*). The Cys-99 variant was fully active, while the Cys-12 variant was completely inactive. Reduction before mixing with P0 cores restored its activity. The results are summarized schematically in Fig. 8. The result with Cys-99 extends the earlier conclusion that the two CTDs of a dimer have considerable freedom to move independently of each other but that they can be locked into disparate proximal orientations, in close contact with each other, with retention of full activity. There is no obligatory, specific functional arrangement or interaction of the two CTDs and there is no functional requirement for the two globular domains to move independently of one another.

The results with the NTD disulfide-linked dimers are consistent with the rhodamine dimer results. The regions around residues 12 and 33 are separated in the bound state. If the amino acid 33 residues are in the putative coiled-coil structure, the result indicates that the residues are facing each other; alternatively, residue 33 may be in a more flexible region that is part of the putative hinge. The former alternative is supported by NMR experiments that show that Ser-33 is in an organized structural region of the dimer (Bushuev et al. 1989). A monoclonal antibody against an epitope destroyed by a cleavage between residues 29 and 30 caused the release of one of the L7/L12 dimers from the ribosome (Sommer et al. 1985;

Tewari et al. 1986). In addition, it has been shown that Phe-30 is involved in the interaction with L10 (Gudkov and Behlke 1978), through which the dimers are attached to the ribosome. It is possible that the Cys-33 cross-link perturbs the structure of the region of L7/L12 responsible for the interaction with the ribosome because of either its direct involvement or an indirect distortion of the binding region. The complete absence of binding of the disulfide-linked dimers and the complete disruption of rhodamine dimers by binding to the ribosome means that the effects pertain to both L7/L12 dimers, even though binding is to nonequivalent sites on L10.

This apparent "plasticity" of the arrangement of the CTDs, or the extent to which they can be deformed, was tested further by making a chimeric construct in which the NTD fragment 1–52:Cys was chemically linked by disulfide interchange to the CTD fragment 53–120:Cys-89 to create a chimeric full-length construct in which the orientation of the globular head was inverted with respect to the attachment to the hinge. This artificial molecule was completely inactive, showing that there is some limit to the plasticity of the CTD conformation with respect to its capacity to support protein synthesis.

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