

# Tetramethylrhodamine Dimer Formation as a Spectroscopic Probe of the Conformation of *Escherichia coli* Ribosomal Protein L7/L12 Dimers\*

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The fluorescent probe tetramethylrhodamine iodoacetamide was attached to cysteine residues substituted at various specific locations in full-length and deletion variants of the homodimeric *Escherichia coli* ribosomal protein L7/L12. Ground-state tetramethylrhodamine dimers form between the two subunits of L7/L12 depending upon the location of the probe. The formation of tetramethylrhodamine 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 different protein variants. Intersubunit tetramethylrhodamine dimers form when tetramethylrhodamine acetamide is attached to two different sites in the N-terminal domain of the L7/L12 dimer (residues 12 or 33), but not when attached to sites in the C-terminal domain (residues 63, 89, or 99). The tetramethylrhodamine dimers do form at sites in the C-terminal domain in L7/L12 variants that contain deletions of 11 or 18 residues within the putative flexible hinge that separates the N- and C-terminal domains. The tetramethylrhodamine dimers disappear rapidly (within 5 s) upon addition of excess unlabeled wild-type L7/L12. It appears that singly labeled L7/L12 dimers are formed by exchange with wild-type dimers. Binding of L7/L12:tetramethylrhodamine cysteine 33 or cysteine 12 dimers either to L7/L12-depleted ribosomal core particles, or to ribosomal protein L10 alone, results in disappearance of the 518-nm absorption band. This result implies a conformational change in the N-terminal domain of L7/L12 upon its binding to the ribosome, or to L10.

Tetramethylrhodamine iodoacetamide (TMRIA)<sup>1</sup> is a sulfhydryl-specific reagent used primarily for fluorescence spectroscopy.

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<sup>1</sup> The abbreviations used are: TMRIA, tetramethylrhodamine iodoacetamide; TMRA, tetramethylrhodamine acetamide; 5'- (or 6'-)TMRIA is a mixture of 5'- and 6'-tetramethylrhodamine iodoacetamide and TMRA represents the same mixture subsequent to reaction with a sulfhydryl and concomitant loss of iodide; L7 and L12 differ only in that the former is acetylated at its N terminus; L7/L12 refers to an unfractionated mixture of the two forms; SE-HPLC, size-exclusion high-performance liquid chromatography; ATP $\gamma$ S, adenosine 5'-(thiotriphosphate).

copy and microscopy. TMRIA is a derivative of rhodamine B which is well known as a quantum counter (1) and as an active medium for lasers (2). It is commercially available from Molecular Probes Inc. (Eugene, OR) as a mixture of the 5' and 6' isomers (Fig. 1). Recently the pure 5' isomer also became available from Molecular Probes. Observations of an absorption band with a maximum at 518 nm associated with high concentrations of rhodamine B in aqueous solutions were reported by Förster and König (3). They attributed this absorption to a ground-state rhodamine B dimer with a dissociation constant ( $K_D$ ) of 667  $\mu$ M. Selwyn and Steinfeld (4) studied the aggregation equilibria of a number of xanthenes dyes and concluded that rhodamine B formed a dimer in aqueous solutions with a dissociation constant of 680  $\mu$ M. Arbeloa and Ojeda (5) extended these studies and determined that neutral and cationic rhodamine B forms ground-state dimers at high concentrations in aqueous solutions ( $K_D = 476$  and 714  $\mu$ M, respectively). Both the Förster and Arbeloa studies reported a ratio of 1.3 for the extinction coefficients at 518 and 555 nm (518/555 ratio) for the rhodamine B dimer and a ratio of 0.4 for the monomer.

Tetramethyl derivatives of rhodamine have also been reported to form ground-state dimers. Ajtai *et al.* (6) reported  $K_D$  values for dimer dissociation of the 5' or 6' isomers of TMRIA as 137 and 56  $\mu$ M, respectively; this study also included absorption spectra of the 5' and 6' isomers of TMRIA indicating that at 500  $\mu$ M the 518/555 ratios reached are less than 1.0. Edmundson *et al.* (7), however, reported a 518/555 ratio for dimers of 5'- and 6'-carboxytetramethylrhodamine of about 1.3.

L7/L12 is a 12-kDa (120 residues) protein present in four copies (in the form of two dimers) in the 50 S subunit of *Escherichia coli* ribosomes, where it is essential for optimal ribosome function in protein biosynthesis (9, 10). A high resolution crystal structure for monomeric C-terminal domain (residues 52–120) has been determined (11). Native L7/L12 possesses no cysteine, but several site-specific cysteine substitution mutants of L7/L12 have been prepared and utilized in cross-linking (12–17) and fluorescence studies (18, 19). Both methods have been used to study the conformation, aggregation state, and ribosomal location of L7/L12. The fluorescence methods essentially provide an average view of the conformation of the protein population on the time scale of nanoseconds, whereas the cross-linking method determines whether two distinct locations can come into contact within a time on the order of minutes.

Time-resolved fluorescence and energy transfer studies (18, 19)<sup>2</sup> in our laboratories have indicated that the two C-terminal domains of L7/L12 possess considerable mobility and are, on

<sup>2</sup> B. D. Hamman, A. V. Oleinikov, G. G. Jokhadze, R. R. Traut, and D. M. Jameson, manuscripts in preparation.

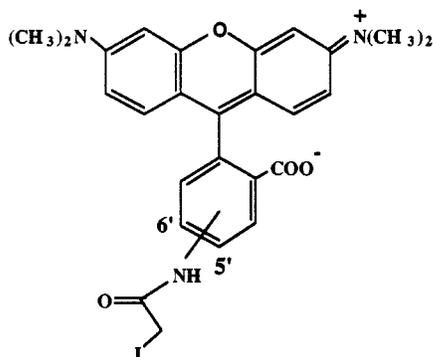


FIG. 1. Structure of 5'-(or 6')-TMRIA.

average, widely separated from one another, even though they can be trapped in high yield by cross-linking as covalently linked disulfide dimers (17). By contrast, these fluorescence studies indicated that the two N-terminal domains (residues 1–33) that are responsible for the dimerization (20), are, on average, closer together. In the present work, the extent of formation of ground-state rhodamine dimers between TMRA bound to the various cysteine locations in full-length or deletion variants of L7/L12, both free and reconstituted into ribosomes, was investigated to extend the findings from cross-linking and fluorescence studies on the conformations of the two domains of the protein.

#### MATERIALS AND METHODS

**Reagents**—TMRIA was used without further purification. All other reagents were analytical grade. Water was distilled and filtered through a Millipore purification system. All experiments subsequent to labeling were performed in the standard buffer 50 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 100 mM KCl, and 3 mM dithiothreitol.

**Proteins**—The genetic construction, expression, purification, and characterization of the L7/L12 variants were as described previously (12, 13, 16, 17). Fig. 2 depicts the putative domain structure of L7/L12 and the locations of the cysteine substitutions utilized in this study and the location of the putative hinge region where deletions were made (we emphasize that this figure is not meant to depict the solution conformation of L7/L12). Only SE-HPLC purified L7/L12 proteins were used in the present study unless otherwise indicated. The full-length variants studied are designated C-12, C-33, C-63, C-89, and C-99; the deletion double variants are designated C-63:Δ35–52 and C-89:Δ42–52. The purification of L10 was described previously (15). Removal of L7/L12 from 70 S ribosomes, and reconstitution of ribosomal cores with L7/L12 variants were accomplished as described previously (21).

**Dye Labeling Procedure**—Modification of the cysteine mutants of L7/L12 with TMRIA was carried out using the methodologies described by Allen (22) except that buffer exchange was accomplished using a Biospin-6 column (Bio-Rad) and unlabeled dye was removed via one Biospin-6 spin followed by one SE-HPLC run. Labeling of the C-63, C-89, or C-99 in the globular domains led to a poor protein recovery. Conversely, proteins labeled at C-12 or C-33 in the α-helical N-terminal domain were recovered in high yield. The reason for the difference in the behavior of proteins labeled within the C- or N-terminal domains is not known. 90–100% labeling efficiencies (see below) were obtained for both the C-terminal and N-terminal domains although the former required labeling in 2 M guanidine hydrochloride or 4 M urea. In these cases the Biospin-6 column used to remove free dye was equilibrated with denaturant at the concentration used for labeling. The deletion variants C-63:Δ35–52 and C-89:Δ42–52 were also labeled to greater than 90% under these conditions. Renaturation and reconstitution of TMRA modified full-length proteins to ribosomal core particles (P0's) showed the same activity in polyphenylalanine synthesis as P0's reconstituted with unmodified wild-type L7/L12, as shown in Table I. Unlabeled hinge-deleted proteins do not support polyphenylalanine synthesis (16).

**Quantification of Probes and Protein**—L7/L12 concentrations were determined colorimetrically using a Coomassie Plus (Pierce) or Bio-Rad assay standardized with gravimetrically quantified L7/L12. The extent of sulfhydryl modification by TMRIA of one of the NTD sites (C-12) and one of the CTD sites (C-89) was determined by reaction with [<sup>14</sup>C]-iodoacetamide before and after the modification by TMRIA. The results,

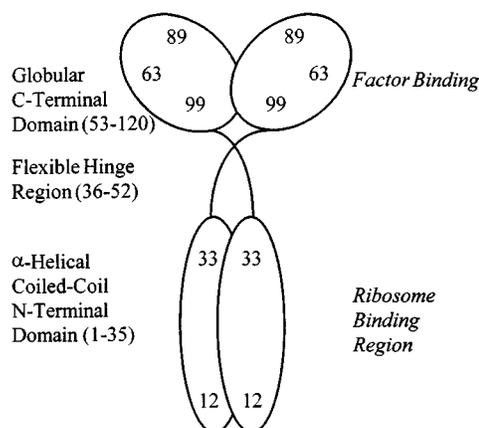


FIG. 2. Schematic diagram of the domain structure of *E. coli* L7/L12 dimer. The C-terminal and N-terminal domains are indicated along with the sites of the various cysteine substitutions and the hinge deletion.

TABLE I  
Activity of ribosomes reconstituted *in vitro* with TMRA labeled L7/L12 variants

Reconstituted particles	Activity ( <sup>14</sup> C]Phe/particle) <sup>a</sup>
1) P0 cores alone	1.0
2) L7/L12 wild-type	14.3
3) L7/L12 TMRA-C-33	13.8
4) L7/L12 TMRA-C-89	14.4
5) L7/L12 TMRA-C-99	13.7

<sup>a</sup> Number of Phe incorporated per 70 S particle per 15 min in poly(U)-directed polyphenylalanine synthesis, reaction carried out as described in Ref. 13.

shown in Table II, demonstrate that the extent of modification approached 100%. All protein concentrations reported here are for dimeric L7/L12.

518/555 ratios of  $1.30 \pm 0.03$  were obtained for the TMRA labeled C-33 or C-12 proteins and this value was taken to represent 100% labeling. The bases of this assumption were the [<sup>14</sup>C]iodoacetamide studies described above and the number of reports in the literature (see Introduction) that the ratio of the two principle absorption bands in such ground-state dimers is approximately 1.3. Addition of excess (15-fold) unlabeled wild-type L7/L12 led to virtually complete disappearance of the TMRA dimer absorption (as evidenced by the resulting 518/555 ratio of 0.41). Subsequent to subunit exchange the absorption at 555 nm was measured and an extinction coefficient for the TMRA monomer bound to the C-12 or C-33 positions was estimated to be  $72,000 \text{ M}^{-1} \text{ cm}^{-1} \pm 2,000$ , assuming 100% labeling. This value may be compared to the extinction coefficient of  $76,000 \text{ M}^{-1} \text{ cm}^{-1}$  given in the Molecular Probes catalogue for the isomeric mixture of TMRIA reacted with β-mercaptoethanol. We note that Corrie and Craik (23) determined an extinction coefficient of  $96,900 \pm 5,300 \text{ M}^{-1} \text{ cm}^{-1}$  at 549 nm for the 5' isomer of TMRIA attached to the thionucleotide ATPγS. The extinction coefficient of TMRA attached to cysteines located within the putative structureless loops (11) of the L7/L12 C-terminal domains (C-63, C-89, and C-99) is the same in buffer or 4 M guanidine hydrochloride (data not shown), indicating negligible effects of secondary or tertiary structure on this property. Assuming that the extinction coefficient of TMRA attached to the C-terminal residues is also approximately  $72,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 555 nm (see above), we can estimate that the labeling ratio obtained for the three C-terminal positions approaches 100%.

**SE-HPLC**—SE-HPLC was carried out on a Tosoh-Hass G2000SW column, using a Perkin-Elmer pump, and a Hewlett-Packard diode array detector and analysis software. The flow rate was 1 ml/min.

**Absorption Spectra**—Absorption spectra were obtained using either a Perkin-Elmer Lambda 5, a Cary 118, or a Varian 1E spectrophotometer.

#### RESULTS

**Formation of TMRA Dimers on Full-length and Hinge-deleted Variants of L7/L12**—Fig. 3 shows the absorption spectra for TMRA conjugated to the C-33 or C-89 positions of dimeric full-length L7/L12. The absorption spectrum for C-33 showed a

TABLE II  
Extent of TMRIA modification

Cys-12 or Cys-89 L7/L12 was reacted with TMRIA as described in the text. After reaction the protein was recovered using Biospin-6 columns and an excess of [<sup>14</sup>C]iodoacetamide was added. Reaction mixtures were incubated for 2 h at room temperature then overnight at 4 °C. The reaction mixtures were then passed over Biospin-6 columns, protein concentrations were determined as described in the text, and radioactivity was determined by liquid scintillation counting. Controls were L7/L12 treated identically but without addition of TMRIA; wild-type L7/L12 treated with [<sup>14</sup>C]iodoacetamide was utilized as a background control.

L7/L12 variant	TMRIA	cpm	[ <sup>14</sup> C]Iodoacetamide incorporated <sup>a</sup>	TMRIA modification
C-12	+	680	0.085	95%
	-	8149	1.008	
C-89	+	537	0.077	96%
	-	6829	0.981	
Wild type	+	317	0.041	
	-	331	0.042	

<sup>a</sup> Picomole/pmol of protein.

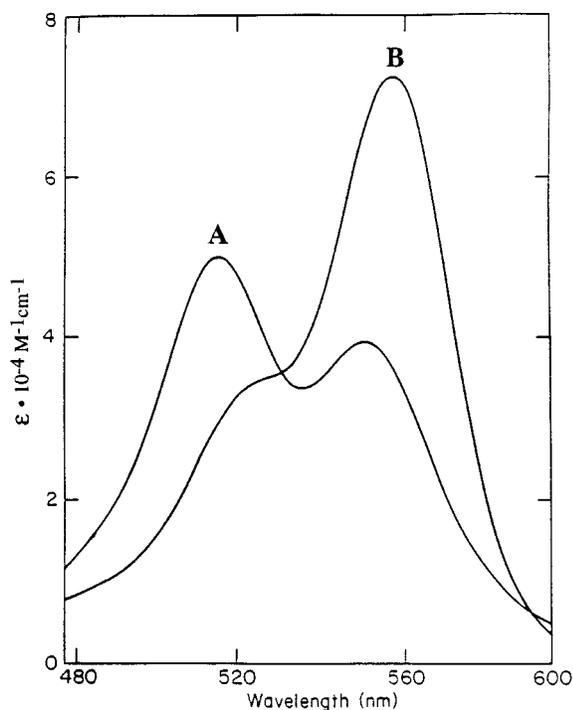


FIG. 3. Extinction coefficients for TMRA bound to L7/L12. Values for the dimeric (A) C-33 and (B) C-89 L7/L12 variants are indicated. The extinction coefficients were based on a value of 72,000 M<sup>-1</sup> cm<sup>-1</sup> at 555 nm for monomeric TMRA and a 518/555 ratio of 1.3 for dimeric TMRA (see "Materials and Methods").

new prominent absorption band at 518 nm, in addition to the 555-nm band characteristic of monomeric rhodamine; the 518/555 ratio in this case was 1.30 ± 0.03. The appearance of the 518-nm band was attributed to the formation of TMRA dimers. Addition of 4 M guanidine hydrochloride to the TMRA-C-33 conjugate resulted in loss of the 518 band and a 518/555 ratio of 0.40 ± 0.01; removal of the denaturant restored the 518-nm band and the 518/555 ratio returned to 1.3. A similar result was obtained for TMRA labeled C-12. These results show that the formation of TMRA dimers, as indicated by the 518-nm band, is dependent on the native structure of the protein.

The absorption spectra for the TMRA labeled C-63 and C-99 conjugates of L7/L12 were essentially identical to that of the C-89 variant (Fig. 3). The spectra of all of the C-terminal domain, full-length variants, however, differed significantly from the absorption spectra corresponding to the TMRA la-

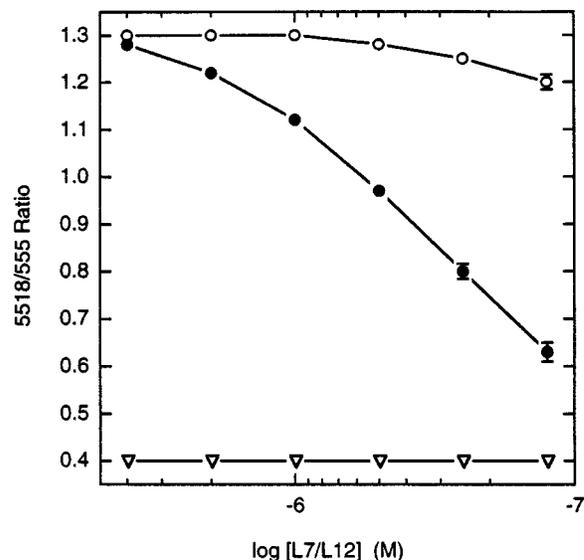


FIG. 4. Dissociation of L7/L12 dimers. Effect of dilution of TMRA labeled C-12 (solid circles), C-33 (open circles), and C-89 (open triangles) L7/L12 on the 518/555 ratio of TMRA absorption. Data represent averaged values from three experiments.

beled C-12 and C-33 variants. Specifically, they exhibited a single prominent absorption band with a maximum at 555 nm and had 518/555 ratios of 0.40 ± 0.01. These spectral characteristics indicated the absence of rhodamine interaction for the probes located at these sites in the C-terminal domain in the full-length protein. However, the behavior of the probes located in the same sites changed in deletion variants of L7/L12 missing either 11 (C-89:Δ42–52) or 18 residues (C-63:Δ35–52) in the hinge linking the C-terminal domain to the N-terminal domain. Specifically, the 518/555 ratios for the TMRA labeled deletion variants were 1.30 ± 0.03, indicating that TMRA dimers form in the CTD sites when the hinge residues were deleted.

*The Dissociation and Exchange of Subunits in L7/L12 Dimers*—The free energy of association of two rhodamines has been reported to be about -4 or -5 kcal/mol (3, 5) and one might expect that this interaction could affect the L7/L12 subunit interaction. Fig. 4 shows the 518/555 ratios for C-12 and C-33 labeled proteins at different dilutions. The TMRA dimers at the C-12 position nearly disappear upon dilution of the protein over the range of 3.3 to 0.1 μM, whereas the TMRA dimers at the C-33 position are still largely intact at 0.1 μM protein. These data are consistent with fluorescence polarization results (19)<sup>2</sup> which indicate dissociation constants for the L7/L12 dimer/monomer equilibrium near 300 and 30 nM for the C-12 and C-33 variants, respectively. Fig. 4 also shows that the 518/555 ratio for the C-89 labeled protein does not change upon dilution. Fluorescence polarization results on this variant, however (data not shown), demonstrate a dimer to monomer dissociation constant near 30 nM.

Fig. 5 shows the effect of addition of increasing quantities of wild-type L7/L12 dimers (up to a 15-fold molar excess) on the 518/555 ratio of TMRA labeled C-33 dimers (1 μM). Addition of equimolar unlabeled wild-type L7/L12 results in a decrease in the 518/555 ratio to 0.81. This result may be compared to the ratio of 0.72 expected for an equilibrium not altered by the TMRA association. The decrease in extinction coefficient at 518 nm of TMRA attached to C-33, subsequent to addition of 10-fold excess wild-type L7/L12, was completed in less than 5 s at 20° C. Wild type L7/L12 that had either been cross-linked with glutaraldehyde to render it incapable of dissociation and exchange, or treated to produce methionine sulfoxides with the

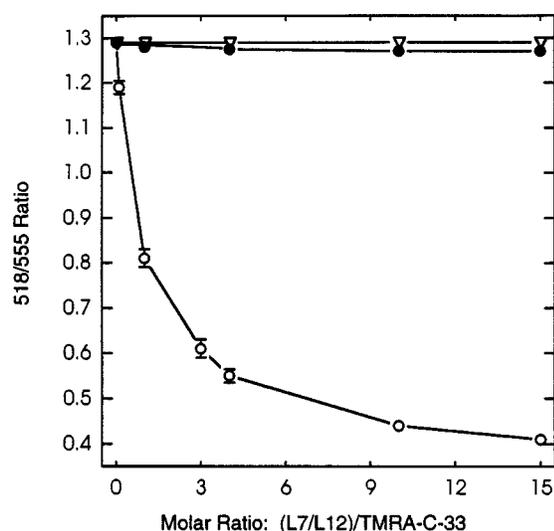


FIG. 5. **Subunit exchange among L7/L12 dimers.** Effect of addition of unlabeled wild-type L7/L12 (open circles), methionine oxidized L7/L12 (open triangles) or glutaraldehyde cross-linked L7/L12 to TMRA labeled C-33. Data represent averaged values from three or more experiments.

resultant formation of L7/L12 monomers (24) incapable of re-association, had no significant effect on the 518/555 ratio (Fig. 5). The retention time of the C-33 TMRA conjugate on the SE-HPLC column corresponded to that observed for unlabeled dimer and was identical in the presence and absence of 10-fold excess wild-type L7/L12 demonstrating the absence of either monomers or higher aggregates (21).

*The Effect of L10 or Ribosomal Core Particles on the Spectroscopic Properties of L7/L12 TMRA Dimers—*Proteins L7/L12 and L10 are known to form a stable pentameric complex containing 2 L7/L12 dimers to a single L10. Table III shows the effect of addition of L10 or 70 S ribosomal cores (lacking L7/L12) on the intersubunit TMRA dimers of C-12 and C-33 labeled L7/L12. The initial concentration of TMRA labeled C-12 and C-33 were  $0.6 \mu\text{M}$ . L10 was titrated with the L7/L12 solution to final concentrations of 0.1, 0.2, 0.3, and  $0.6 \mu\text{M}$ , and absorption spectra were determined after each addition. The decrease in the 518/555 ratio with increasing L10 concentration indicates that the TMRA dimers dissociate upon binding of the L7/L12 (C-12 or C-33) to L10. Addition of ribosomal cores lacking L7/L12 had the same effect on the 518/555 ratio, although corrections for light scattering reduced the precision of this determination.

#### DISCUSSION

The formation of TMRA ground-state dimers in L7/L12 cysteine substituted variants conjugated with the sulfhydryl specific probe was used to investigate conformational aspects of the C-terminal and N-terminal domains of L7/L12 dimer in solution. The formation of the new 518-nm absorbance peak characteristic of the rhodamine-rhodamine interaction and the ratio of the intensities of the new 518-nm band to the pre-existing 555-nm band served as a convenient way of quantifying the spectroscopic data. Probes situated at two locations in the N-terminal domain both displayed the 518-nm band, whereas probes in the C-terminal domain did not. This observation is consistent with mobility of the CTD's inferred from  $^1\text{H}$  NMR (25, 26), ESR (27), and electron microscopy (28) studies and with energy transfer studies on fluorescein and AEDANS attached to the C-63 and C-89 positions which indicated that, on average, the C-63 to C-63 or the C-89 to C-89 intersubunit distances are  $>80 \text{ \AA}$  (19).<sup>2</sup> Despite the absence of close proxi-

TABLE III  
518/555 ratios for TMRA-C-33 or C-12 in the presence of L10 or ribosome cores (standard deviations in the L10 cases were less than 0.03)

Sample	518/555 Ratio
$0.6 \mu\text{M}$ TMRA-C-33	1.28
+ $0.1 \mu\text{M}$ L10	1.03
+ $0.2 \mu\text{M}$ L10	0.72
+ $0.3 \mu\text{M}$ L10	0.53
+ $0.6 \mu\text{M}$ L10	0.51
$0.6 \mu\text{M}$ TMRA-C-12	1.01
+ $0.3 \mu\text{M}$ L10	0.49
+ $0.6 \mu\text{M}$ L10	0.48
$0.6 \mu\text{M}$ TMRA-C-33	1.30
+ $0.3 \mu\text{M}$ 70 S cores	0.49
+ $0.6 \mu\text{M}$ 70 S cores	0.48
$0.6 \mu\text{M}$ TMRA-C-12	1.02
+ $0.3 \mu\text{M}$ 70 S cores	0.47
+ $0.6 \mu\text{M}$ 70 S cores	0.46

imity of the two C-terminal domains, covalent disulfide cross-linked complexes have been obtained in high yield for the C-63, C-89 (19), and C-99<sup>3</sup> positions suggesting that, on the time scale of the cross-linking experiments, these domains are mobile and can approach close enough to permit disulfide bond formation. When the hinge region linking the two domains is substantially shortened in deletion variants (C-63: $\Delta$ 35–52 and C-89: $\Delta$ 42–52), TMRA dimer formation takes place for probes in the C-terminal domain. This observation implies that the hinge facilitates the mobility and separation of the two C-terminal domains in the full-length protein.

TMRA attached to N-terminal sites 12 and 33, both within the known dimerization domain, formed rhodamine dimers in the free proteins with absorption properties closely resembling those of dimers formed by free dye at high concentrations (518/555 ratios of 1.3). This result is consistent with the parallel non-staggered model of the L7/L12 dimer as depicted in Fig. 2. It also suggests that both the 5' and 6' isomers of the probe were able to form dimers; however, it is also possible that one isomer in the mixture of 5' and 6' reacts preferentially with the target cysteine. Ajtai *et al.* (6) suggested that the TMRIA from Molecular Probes contains mainly the 6' isomer and also suggest differential reactivity of the two isomers.

The 518-nm absorption band of TMRA labeled C-12 or C-33 dimers disappeared rapidly (within 5 s) upon addition of excess unlabeled wild-type L7/L12. These results indicate that subunit exchange can occur among the L7/L12 dimer population and that the 518-nm absorption band of the TMRA dimers provide a convenient spectroscopic handle to monitor this process. These results are consistent with similar observations on rapid subunit exchange in other dimeric and tetrameric systems (29–32).

The experiments revealed differences in the two NTD sites implying different orientations of the two polypeptide chains in the C-12 and C-33 regions. Specifically, the dissociation constant for the TMRA labeled C-12 dimer/monomer equilibrium is near 300 nM (judged by the midpoint of the decrease in the 518/555 ratio upon dilution), in good agreement with results of fluorescence polarization studies on the dissociation of C-12 variants labeled with either one or two fluorophores per L7/L12 dimer (19).<sup>4</sup> These fluorescence polarization studies also indicated a dissociation constant for singly and doubly labeled C-33

<sup>3</sup> A. V. Oleinikov, G. G. Jokhadze, and R. R. Traut, unpublished observations.

<sup>4</sup> B. D. Hamman and D. M. Jameson, unpublished results.

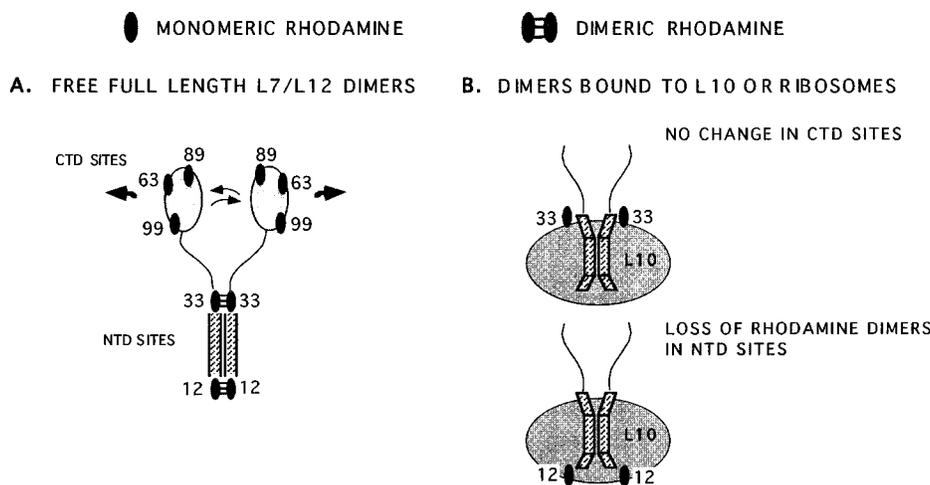


FIG. 6. Schematic diagram summarizing the formation and loss of TMRA dimers. Results are summarized for full-length L7/L12 dimers, both free and bound to L10 or 70 S ribosomal core particles. The arrows indicate mobility of the C-terminal domains.

variants near 30 nm, a value consistent with the small change observed in the 518/555 ratio for TMRA labeled C-33 over the dilution range accessible by absorption measurements (Fig. 4). These results and the 518/555 ratio observed for equimolar, subunit exchanged mixtures of unlabeled wild-type L7/L12 and L7/L12:TMRA-C-33 suggest that TMRA dimer formation does not significantly perturb the L7/L12 dimer/monomer equilibrium. Conformational alterations of different magnitude in the regions surrounding residues 12 or 33 may be necessary for the TMRA-TMRA interaction, and the free energy of the TMRA dimer formation may compensate for the free energy loss associated with such alterations. Alternatively, microscopic details of the environment around the TMRA moieties, such as the dielectric constant and solvation, may differ sufficiently from the bulk solvent to alter the free energy of formation of a ground-state complex, and may be different for the probes at the two locations. Different orientations for the TMRA molecules in the ground-state dimer have been proposed (5-7) but our results do not address this issue. The reasons for the difference in the dimer/monomer equilibria for the C-12 and C-33 variants is not presently clear.

The dissociation of the TMRA dimers, located at both residues C-12 or C-33, upon binding of L7/L12 to L10, suggests that a conformational change in the N-terminal domain of L7/L12 accompanies the binding. Residue 12 is near the N-terminal end of the domain, while residue 33 is near the junction with the putative hinge region. This observation is consistent with the finding that dimers of L7/L12 oxidatively cross-linked in solution by disulfide bonds between the two C-33 or C-12 residues fail to bind to ribosomal core particles (17)<sup>3</sup> which suggests that cross-linking prevents conformational changes at both sites in the N-terminal domain required for or accompanying binding to L10. Protein L10 is the major ribosomal component that anchors both L7/L12 dimers to the ribosome, and it is not surprising that ribosomal core particles lacking only L7/L12, but retaining L10, have the same effect on TMRA dimers as does free L10. The effect must pertain to both L7/L12 dimers even though they bind to non-equivalent sites in L10. These observations are summarized in schematic form in Fig. 6. The left hand image in Fig. 6A summarizes the locations of all five rhodamine conjugates, and indicates the formation of rhodamine dimers in the two N-terminal locations, but not in either of the three C-terminal locations, the mobility and separation of which are indicated by arrows. The right-hand images in Fig. 6B show the conformational perturbation and disruption of the two N-terminal sites accompanying binding to

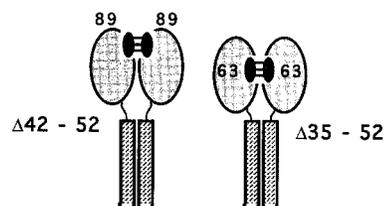


FIG. 7. Schematic diagram indicating TMRA dimer formation in hinge-deleted L7/L12 variants.

the ribosomal core particle or to purified L10.

Fig. 7 shows the effect of the deletion of residues in the hinge region on TMRA dimer formation at two of the sites in the C-terminal domain. Extensive studies have not yet been completed in which each of the three CTD sites have been combined with both the longer and shorter deletions. TMRA dimers form at sites 63 and 89, with the longer and shorter deletions, respectively. The result suggests that the loss of flexibility resulting from the hinge deletions facilitates stable interaction between the probes. The magnitude of the 518-nm band in these experiments indicates that the entire population undergoes this interaction, despite the fact that residues 63 and 89 are located in exposed loops facing in different directions, according to the x-ray structure of C-terminal domain monomers (11). The ability of cysteine residues in these two locations (17) and also at residue 99<sup>3</sup> to form zero-length disulfide cross-links has been shown previously. These results suggested flexibility in the orientation as well as the proximity of the two C-terminal domains. The present results suggest that, even when the hinge is shortened, sufficient flexibility remains to bring either the two 63 loops, or the two 89 loops into proximity. The dynamics of the C-terminal domain and its interaction with the N-terminal domain and L10 are being studied by energy transfer and time-resolved fluorescence methods.

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