

## Dimer/Monomer Equilibrium and Domain Separations of *Escherichia coli* Ribosomal Protein L7/L12<sup>†</sup>

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**ABSTRACT:** The dimer to monomer equilibrium and interdomain separations of cysteine variants of L7/L12 have been investigated using fluorescence spectroscopy. Steady-state polarization measurements on cysteine containing variants of L7/L12, labeled with 5-(iodoacetamido)fluorescein, demonstrated dimer to monomer dissociation constants near 30 nM for variants labeled at position 33, in the N-terminal domain, and positions 63 and 89, in the C-terminal domain. A dissociation constant near 300 nM was determined for a variant labeled at position 12, in the N-terminal domain. The polarization of a labeled C-terminal fragment did not change over the range of 200  $\mu$ M to 1 nM, indicating that this construct remains monomeric at these concentrations, whereas a dimer to monomer dissociation constant near 300 nM was observed for an FITC labeled N-terminal fragment. Intersubunit fluorescence resonance energy self-transfer was observed when appropriate probes were attached to cysteines at residues 12 or 33, located in the N-terminal domain. Probes attached to cysteines at positions 63 or 89 in the C-terminal domain, however, did not exhibit intersubunit self-transfer. These results indicate that these residues in the C-terminal domains are, on average, separated by greater than 85 Å. Intersubunit self-transfer does occur in a C-89 double mutation variant lacking 11 residues in the putative hinge region, indicating that the loss of the hinge region brings the two C-terminal domains closer together. Rapid subunit exchange between unlabeled wild-type L7/L12 and L7/L12 variants labeled in the N-terminal domain was also demonstrated by the loss of self-transfer upon mixing of the two proteins.

L7/L12,<sup>1</sup> a 12 kDa (120 residues) protein present in four copies (in the form of two dimers) in the 50S subunit of *Escherichia coli* ribosomes, is essential for ribosome function in protein biosynthesis (Möller et al., 1983; Casiano et al., 1990). Native L7/L12 possesses no cysteine, but several variants of L7/L12 containing cysteine at specific residues have been prepared and characterized by cross-linking (Zecherle et al., 1992a,b; Marakov et al., 1993; Traut et al., 1993) and spectroscopic methods including both fluorescence (Hamman, 1994; Hamman et al., 1996b,c) and absorption spectroscopy (Hamman et al., 1996a). Cross-linking and spectroscopic methods have been used to study the confor-

mation, state of aggregation, and ribosomal location of L7/L12. L7/L12 is generally described as a stable dimer, but few studies quantitatively address the relevant oligomeric equilibria. Kar and Aune (1981) described analytical ultracentrifugation experiments which they interpreted in terms of monomer/dimer/tetramer equilibria characterized by equal monomer/dimer and dimer/tetramer association constants of  $3.5 \times 10^4 \text{ M}^{-1}$ . Conversely, sedimentation equilibrium (Möller et al., 1972; Luer & Wong, 1979, 1980), chemical cross-linking (Österberg et al., 1976), low-angle X-ray scattering (Österberg et al., 1976), and disulfide cross-linking (Traut et al., 1993) studies have all indicated that L7/L12 is >90% dimeric at micromolar and submicromolar concentrations. Gudkov and co-workers (1978, 1995) have suggested that the N-terminal domain of L7/L12 is responsible for the dimerization, and a coiled-coil type interaction has been proposed (Tsurugi & Mitsui, 1991). Evidence for a parallel alignment of the L7/L12 subunits was found by Oleinikov et al. (1994) using zero-length cross-linking methods and also by a study of tetramethylrhodamine dimer formation (Hamman et al., 1996a). Recently, Gudkov et al. (1996) reported NMR data on L7/L12 in solution which also indicated a parallel (head-to-head) orientation of the subunits and suggested that the N-terminal domain is comprised of an antiparallel four  $\alpha$ -helix bundle.

Fluorescence methodologies have often been utilized to study equilibria involving protein–ligand and protein–protein interactions. Fluorescence polarization is particularly useful in this regard since the method reports on changes in the hydrodynamics of an appropriately labeled system and can be used to quantify the state of oligomerization under

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<sup>1</sup> Abbreviations: 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; 5-IAF, 5-(iodoacetamido)fluorescein; 1,5-IAEDANS, 5-[[[(2-(iodoacetyl)amino)ethyl]amino]naphthalene-1-sulfonate]; FITC, fluorescein isothiocyanate; TMRIA, tetramethylrhodamine iodoacetamide; TMRA, tetramethylrhodamine acetamide; 5-AF and 1,5-AEDANS refer to the probes after reaction with the protein and loss of iodide; CTF, C-terminal fragment, residues 52–120, of L7/L12; NTF, N-terminal fragment, residues 1–53, of L7/L12; SE-HPLC, size-exclusion high-performance liquid chromatography; homo-FRET, fluorescence resonance energy transfer between identical fluorophores, i.e., self-transfer.

equilibrium conditions (Weber, 1992; Jameson & Sawyer, 1995). In the present study, we have taken advantage of the fact that L7/L12 lacks cysteine residues to selectively introduce single cysteines at specific locations along the peptide chain. Suitable sulfhydryl reactive fluorescence probes may then be attached to these cysteine residues and used to study both the state of aggregation and dynamic aspects of the protein. Interpretation of polarization results often require time-resolved measurements, and such determinations were carried out and presented in the previous paper (Hamman et al., 1996c). The time-resolved fluorescence results described in the previous paper demonstrated that probes attached to L7/L12 exhibit varying degrees of local mobility depending upon their location. Probes attached to the C-terminal domain, specifically in positions C-63 and C-89 (Hamman et al., 1996c), and C-99 (Hamman, 1994), exhibit extensive depolarization due to local probe mobility as well as independent mobility of the two C-terminal domains. Probes attached to the N-terminal domain, specifically positions C-33 and C-12, exhibit global rotational relaxation times consistent with the molecular mass of the dimeric N-terminal domain. These observations have implications for the changes in polarization observed when the labeled L7/L12 passes from the dimeric to the monomeric state.

We have addressed the question of subunit exchange between labeled and unlabeled L7/L12 dimers in addition to determination of the dimer/monomer equilibrium. Several groups have recently reported studies on rapid subunit exchange among populations of multimeric proteins (Erijman & Weber, 1991, 1993; Fekkes et al., 1995; Chung & Seifried, 1995). Erijman and Weber (1991, 1993), for example, used fluorescence depolarization due to fluorescence resonance energy self-transfer (homo-FRET) to study the rate of exchange of subunits in tetrameric proteins. In the present study, we demonstrate that both fluorescein and AEDANS probes attached to positions C-33 and C-12 of L7/L12 experience self-transfer (homo-FRET) when both subunits are labeled and that addition of unlabeled L7/L12 leads rapidly to exchange of subunits among the labeled and unlabeled populations, resulting in loss of homo-FRET. These homo-FRET studies also address the issue of the average intersubunit distance between specific residues of L7/L12.

## MATERIALS AND METHODS

**Buffers, Proteins, and Ribosomes.** The construction, expression and purification of the recombinant L7/L12's were described in the preceding paper and references therein (Hamman et al., 1996c). Monomers of L7/L12 were formed from methionine oxidation as previously described (Caldwell et al., 1978; Gudkov & Behlke, 1978), and purification of oxidized monomers was accomplished by gel filtration HPLC as described in the previous paper (Hamman et al., 1996c). Disulfide cross-linked dimers of the cysteine variants of L7/L12 were formed as previously described (Zecherle et al., 1992a,b).

**Labeling, Purification, and Quantification.** All fluorescent probes were purchased from Molecular Probes, Inc. (Junction City, OR), and used without further purification. The protocol used to label the L7/L12 variants with sulfhydryl specific probes was a modification of a published method

(Allen, 1981) as described in the previous paper (Hamman et al., 1996c). Egg white lysozyme (Sigma) was labeled for 15 min with a 30-fold molar excess of FITC in a carbonate buffer (pH 9.4), and excess unreacted probe was removed as described in the previous paper. The extinction coefficients utilized for protein bound probes were  $70\,000\text{ M}^{-1}\text{ cm}^{-1}$  (5-AF; 488 nm),  $5500\text{ M}^{-1}\text{ cm}^{-1}$  (1,5-AEDANS; 351 nm), and  $71\,000\text{ M}^{-1}\text{ cm}^{-1}$  (FITC; 488 nm). The concentration of L7/L12 was determined by either a Coomassie Plus (Pierce Co.) or Bio-Rad (Bio-Rad Co.) assay. Standards were either BSA or gravimetrically quantified L7/L12. All protein concentrations reported here are for dimeric L7/L12. Labeling efficiencies, based on the appropriate absorbances and extinction coefficients of the bound fluorophores and the measured protein concentrations, were routinely in the range of 95–100%; nonspectroscopic verification of labeling ratios of L7/L12 with fluorescent sulfhydryl probes has been carried out previously using a [ $^{14}\text{C}$ ]iodoacetamide method which corroborated the spectroscopic based determinations (Hamman et al., 1996a).

**Polarization Measurements.** Steady-state polarization measurements were carried out using an SLM 8000 spectrofluorimeter (SLM-Aminco, Inc., Champaign, IL). Excitation of the fluorescein adducts was at 475 nm, and emission at wavelengths greater than 515 nm was viewed through Schott 085 cut-on filters. Background due to scattered light or buffer fluorescence was insignificant down to 20 nM fluorescein; below this level, blank subtraction was utilized. For AEDANS adducts, excitation was at 351 nm and emission was viewed through Schott KV399 cut-on filters which passed wavelengths greater than 380 nm. Concentrations were kept above 200 nM to eliminate the necessity for background corrections. In all cases, measurements were averaged until a standard deviation of 0.001 was obtained. In most cases, a dual path length cuvette (10 mm by 2 mm) was utilized with the emission viewed through the shorter path; at higher fluorescein concentrations ( $>10\ \mu\text{M}$ ) a 2 mm by 2 mm cuvette was utilized to minimize inner filter effects. The dissociation constants reported are averages derived from results on four or more preparations for the C-63, C-89, and C-33 variants. For other proteins, the results represented the averages of three or more different preparations.

**Homo-FRET Measurements.** Fluorophores which exhibit small Stokes shifts, such as fluorescein, can effectively undergo self-transfer which is readily detected by the resulting depolarization of the emission (Gaviola & Pringsheim, 1924; Weber, 1960). The efficiency of energy transfer in cases where the fluorophores are isotropically distributed can be calculated from a knowledge of the polarization in the absence and presence of energy transfer as demonstrated by Weber (1954, 1960). In our case of one acceptor–donor pair, the appropriate expressions for the decay of the intensity parallel and perpendicular to the excitation direction in the presence of energy transfer are:

$$I(t)_{\text{par}} = (I_0/3)(1 + r_{01}(1 + e^{-Kt}) + r_{02}(1 - e^{-Kt}))e^{-\Gamma t} \quad (1)$$

$$I(t)_{\text{perp}} = (I_0/3)(1 + r_{01}(1 + e^{-Kt})/2 - r_{02}(1 - e^{-Kt})/2)e^{-\Gamma t} \quad (2)$$

where  $I_0$  is the initial intensity,  $r_{01}$  and  $r_{02}$  are, respectively, the anisotropy decays of the donors and acceptors only,  $\Gamma$  is

the fluorescence decay rate, and  $K$  is the rate of transfer between donor and acceptor. These expressions lead, upon integration, to a steady-state expression for the efficiency of energy transfer ( $E$ ) as a function of the anisotropy in the presence and absence of acceptor.

$$E = 2(r_{01} - \langle r \rangle) / (r_{01} - r_{02}) \quad (3)$$

where  $\langle r \rangle$  is the observed anisotropy. In the case of random orientation, i.e.,  $3 \cos^2 \Theta - 1 = 0$ ;  $r_{02} = 0$  and then  $E = 2(r_{01} - \langle r \rangle) / r_{01}$ . This case corresponds to the well-known approximation of  $\kappa^2 = 2/3$ , where  $\kappa^2$ , the orientation factor, is given by:

$$\kappa^2 = (\cos \Theta_{12} - 3 \cos \Theta_1 \cos \Theta_2)^2 \quad (4)$$

where  $\Theta_{12}$  is the angle between the direction of the two transition dipole moments and  $\Theta_1$  and  $\Theta_2$  are the angles of the two dipoles with the line joining the center of the dipoles. As pointed out by Weber (Weber, 1960; Weber & Shinitzky, 1970), homo-FRET disappears upon excitation at the red edge of the absorption spectrum.  $E$  is in turn related to  $R_0$ , the critical distance for 50% energy transfer, and  $R$ , the distance between donor and acceptor, by the following equation (Förster, 1948):

$$E = R_0^6 / (R_0^6 + R^6) \quad (5)$$

where  $R_0$  is calculated as follows:

$$R_0 = 9780(n^{-4} Q \kappa^2 J)^{1/6} \quad (6)$$

where  $n$  is the refractive index (in most buffers taken as 1.4),  $Q$  is the quantum yield of the donor in the absence of acceptor,  $\kappa^2$  is the orientation factor, and  $J$  is the overlap integral between donor emission and acceptor absorption as calculated by:

$$J = \sum F_d(\lambda) \epsilon_a(\lambda^4 \Delta\lambda) / (\sum F_d(\lambda) \Delta\lambda) \quad (7)$$

where  $F_d(\lambda)$  is the fluorescence intensity of the donor at wavelength  $\lambda$ , and  $\epsilon_a(\lambda)$  is the extinction coefficient of the acceptor at wavelength  $\lambda$ . In the present case, the summation was carried out using 5 nm intervals ( $\Delta\lambda = 5$  nm); emission spectra were corrected for instrument response parameters.

**Immunoaffinity Chromatography.** Polyclonal anti-fluorescein antibodies were a generous gift from Dr. Edward Voss, Jr. The antibodies were conjugated to cyanogen bromide activated Sepharose 4B as described by Harlow and Lane (1988).

## RESULTS

**Concentration Dependent Dissociation of 5-AF Labeled Variants of L7/L12.** The polarizations of 5-AF attached to both C- and N-terminal domains of L7/L12, as a function of protein concentration, are shown in Figures 1 and 2. The polarization values at the highest protein concentrations vary depending upon the location of the label as discussed in the previous paper (Hamman et al., 1996c). In addition to depolarization due to rotational mobility, depolarization can also result from homo-FRET as shown in Figure 1 for the case of fluorescein labeled C-33. The polarization values at the highest protein concentrations for the C-33-F adducts were significantly higher when singly labeled L7/L12 dimers

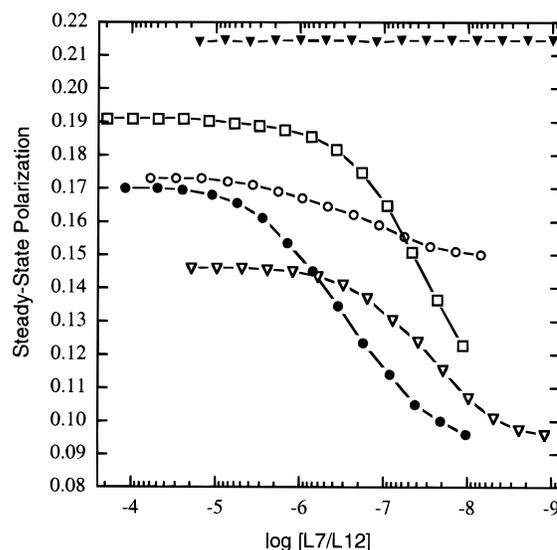


FIGURE 1: Concentration dependence of the polarization of L7/L12 variants modified in the N-terminal domain. Filled triangles represent a control, FITC labeled lysozyme (1.8 fluoresceins per protein); open triangles represent doubly labeled C-33-F; open squares represent singly labeled C-33-F (exchanged with a 15-fold molar excess of unlabeled wild-type L7/L12); filled circles represent singly labeled C-12-F (exchanged with an 8-fold molar excess of unlabeled and reduced C-12); open circles represent FITC labeled NTF.

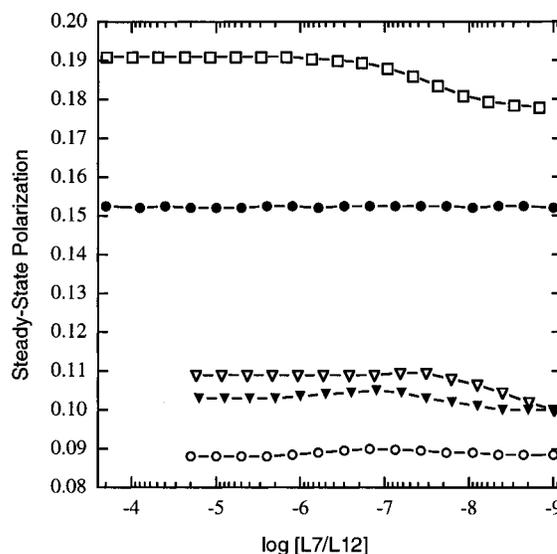


FIGURE 2: Concentration dependence of the polarization of L7/L12 variants modified in the C-terminal domain. Open squares represent C-89-F; filled triangles represent C-63-F; open circles represent C-99-F; open triangles represent C-89:Δ42-52-F; filled circles represent FITC labeled CTF.

were formed by subunit exchange, due to the resulting loss of self-transfer between the fluorescein moieties located on adjacent subunits. Both singly and doubly labeled C-33 exhibited concentration dependent polarization values indicative of dimer to monomer dissociation. In the case of L7/L12 labeled with fluorescein at positions C-63 or C-89, singly or doubly labeled dimers gave the same polarization values indicating the absence of homo-FRET. A small extent of homo-FRET was observed for the C-99 case. In all these C-terminal domain cases, the variation of the polarization as a function of protein concentration was much less dramatic than observed for N-terminal labels. No change in polarization was observed for C-89-F CTF over the range of 200

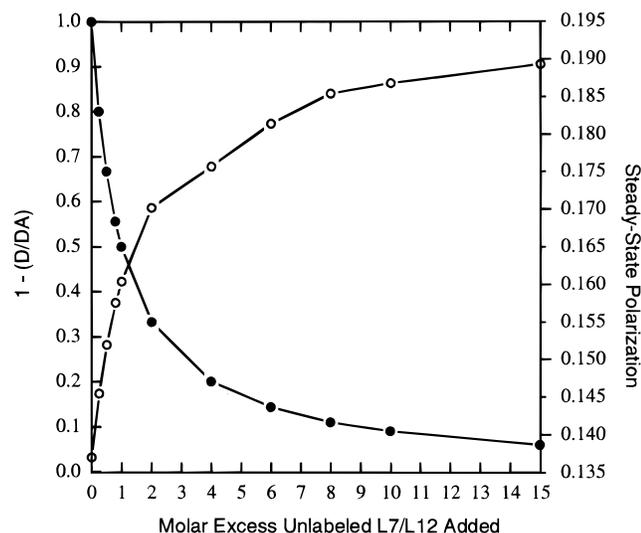


FIGURE 3: Effect of adding unlabeled wild-type L7/L12 to C-33-F. Excitation wavelength was 475 nm. Open circles represent the observed polarization; filled circles represent the fraction of total emission from singly labeled dimers (D) to that of doubly labeled dimers (DA).

$\mu\text{M}$  to 1 nM, whereas a concentration dependent decrease in polarization occurred in the case of FITC labeled NTF, indicative of a dimer to monomer transition. Dimer/monomer dissociation constants were calculated using the additivity of anisotropy principles (Weber, 1952; Jameson & Sawyer, 1995).

*Subunit Exchange of L7/L12 As Revealed by Homo-FRET and Chromatography.* A rapid (half-time less than 5 s) increase in polarization of the 5-AF labeled C-33 variant was observed after addition of excess unlabeled wild-type L7/L12. This observation was consistent with the previously reported (Hamman, et al., 1996a) rapid disappearance of ground-state tetramethylrhodamine (TMRA) dimers, which formed between the C-33 or C-12 positions in TMRA labeled L7/L12 variants, upon addition of unlabeled L7/L12. Figure 3 shows that the polarization of 5-AF attached to C-33 increases from 0.138 to 0.160 upon addition of equimolar unlabeled wild-type L7/L12. Addition of a 10-fold molar excess of unlabeled wild-type L7/L12 results in a further increase in the polarization to 0.187. Increases in polarization after addition of unlabeled L7/L12 were also observed with the C-12 and C-99 samples but not for C-63 or C-89 due to the lack of homo-FRET for these adducts. Subunit exchange of C-63 or C-89 variants was demonstrated by immunoaffinity chromatography. TMRA labeled L7/L12 was incubated with Sepharose 4B linked to anti-fluorescein antibodies in the absence and presence of fluorescein labeled L7/L12. The results showed that significant amounts of TMRA labeled L7/L12 were bound to the antibody labeled Sepharose 4B only when fluorescein labeled L7/L12 was present, indicating that subunit exchange had occurred (Table 1).

*Estimation of Intersubunit Domain Distances by Homo-FRET.* The homo-FRET phenomenon can be used to estimate the average distance between fluorescein moieties on adjacent subunits. A clear demonstration that depolarization due to homo-FRET occurs in the doubly labeled C-33-F can be found in the excitation polarization spectra shown in Figure 4. The signature of homo-FRET is the loss of energy transfer upon excitation at the red-edge of the

Table 1: Binding of Various L7/L12 Adducts to Anti-Fluorescein Immunoabsorbant<sup>a</sup>

sample added to immunoabsorbant	% of AF fluorescence bound to immunoabsorbant	% of TMRA fluorescence bound to immunoabsorbant
0.3 (or 0.6) $\mu\text{M}$ C-33-F	95	
0.3 (or 0.6) $\mu\text{M}$ C-33-TMRA		10
0.3 $\mu\text{M}$ C-33-F exchanged with 0.3 $\mu\text{M}$ C-33-TMRA		55
0.3 (or 0.6) $\mu\text{M}$ C-89-F	96	
0.3 (or 0.6) $\mu\text{M}$ C-89-TMRA		11
0.3 $\mu\text{M}$ C-89-F exchanged with 0.3 $\mu\text{M}$ C-89-TMRA		58

<sup>a</sup> In each case, the fluorescence spectrum of 250  $\mu\text{L}$  of protein/dye conjugate was measured, and the sample was then incubated with 250  $\mu\text{L}$  of immunoabsorbant at room temperature with gentle mixing. The immunoabsorbant was pelleted for 2 min in a microcentrifuge at maximum speed, and the fluorescence spectrum of the supernatant was immediately determined. Spectral areas, corrected for dilution, were used to quantify the fluorescence. Fluorescein was excited at 485 nm, and rhodamine was excited at 560 nm (where fluorescein does not absorb). The values given represent averages of three separate experiments, and standard errors are  $\pm 6\%$ .

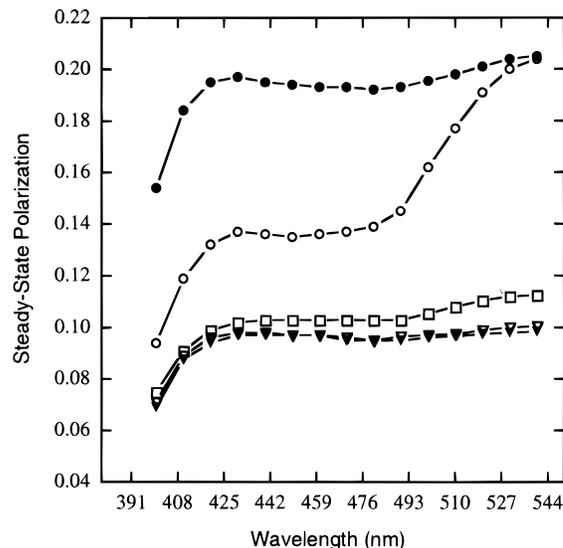


FIGURE 4: Excitation polarization spectra for various C-33-F cases. Open circles represent 1  $\mu\text{M}$  C-33-F; filled circles represent 1  $\mu\text{M}$  C-33-F mixed with 15  $\mu\text{M}$  unlabeled wild-type L7/L12; open squares represent 8 nM C-33-F; open triangles represent 1  $\mu\text{M}$  methionine oxidized C-33-F; filled triangles represent 1  $\mu\text{M}$  C-33-F in the presence of 4 M guanidine hydrochloride.

absorption, as demonstrated by Weber (Weber, 1960; Weber & Shinitzky, 1970). As shown in Figure 4, doubly labeled C-33-F is characterized by a nearly constant polarization value (near 0.14) upon excitation at wavelengths between about 430 and 475 nm, but as the excitation wavelength exceeds 475 nm, the polarization begins to rise conspicuously, reaching a value near 0.2 upon 530 nm excitation. This excitation polarization spectrum contrasts with that observed for singly labeled (after exchange with 15-fold excess unlabeled wild-type L7/L12) C-33-F which maintains a higher polarization over the excitation range of 430–475 nm (near 0.20) and which rises only slightly above 475 nm. Monomeric C-33-F, obtained by methionine oxidation treatment or by addition of 4 M GuHCl, demonstrated a lower and nearly constant polarization over the excitation range

Table 2: Anisotropies in the Presence and Absence of Homo-FRET in L7/L12 Adducts: Estimation of Maximum Interprobe Distances<sup>a</sup>

	$\langle r \rangle$	$r_{01}$	$E$	$\langle R_0 \rangle$ (Å)	$\langle R \rangle_{\text{dyn}}$ (Å)
(A) 5-Acetamidofluorescein					
C-12	0.088	0.131	0.66	40	37
C-33	0.096	0.138	0.61	40	38
C-63	0.072	0.072	0.00	40	>90
C-89	0.135	0.136	0.01	41	88
C-89:Δ42–52 <sup>b</sup>	0.074	0.102	0.54	38	39
C-99	0.065	0.074	0.24	40	46
(B) AEDANS					
C-12	0.049	0.053	0.15	11	14
C-33	0.051	0.057	0.21	11	13
C-63	0.040	0.040	0	11	>22
C-89	0.049	0.049	0	11	>22
C-99	0.039	0.039	0	11	>22

<sup>a</sup> Where  $\langle r \rangle$  and  $r_{01}$  are the anisotropies observed before and after subunit exchange, respectively,  $E$  is the efficiency of energy transfer calculated using eq 3 (with  $r_{02} = 0$ ),  $\langle R_0 \rangle$  is the distance for 50% transfer efficiency calculated from the absorption and emission spectra of the probes as described in the text, and  $\langle R \rangle_{\text{dyn}}$  is the calculated distance of the probes assuming dynamic averaging, i.e.,  $\kappa^2 = 2/3$ . Standard deviations for the anisotropy determinations were  $\pm 0.001$ , and the errors associated with the distance estimates are less than  $\pm 6$  Å in all cases.

<sup>b</sup> Subunit exchange was accomplished in this case after treatment of the sample with 4 M GuHCl in the presence of 15-fold excess unlabeled C-89:Δ42–52, followed by removal of the GuHCl by Biospin 6 filtration; treatment of C-89:Δ42–52 with GuHCl but without addition of unlabeled protein did not affect the anisotropy.

of 430–530 nm. To estimate the efficiency of homo-FRET, we compare the polarizations (or the analogous anisotropies ( $r$ ), where  $r = 2P/(3 - P)$ ) observed for the case of doubly and singly labeled proteins upon excitation at 475 nm. In the case of C-33-F, the anisotropy values are 0.096 and 0.138, which correspond to  $\langle r \rangle$  and  $r_{01}$ , respectively, in eq 3. The anisotropy values for other dimers, doubly and singly labeled with fluorescein, are given in Table 2A. The behaviors of C-12, C-33, C-99, and C-89:Δ42–52 are distinctly different from those of C-63 or C-89. In the latter cases, the anisotropies of dimers, either doubly or singly labeled with fluorescein, are the same, whereas in the former cases these values are significantly different.

The critical distance ( $R_0$ ) for energy transfer between AEDANS molecules is calculated to be less ( $\sim 11$  Å) than that for the case of fluorescein ( $\sim 40$  Å) due to the relatively small overlap integral (eq 7) between AEDANS' absorption and emission. Nonetheless, as shown in Table 2B, a small extent of homo-FRET was evident in the case of AEDANS labeled C-12 and C-33, but not in the C-63, C-89, or C-99 cases.

## DISCUSSION

The concentration dependence of the polarization of fluorescein labeled full-length L7/L12 indicates a dimer/monomer dissociation constant near 30 ( $\pm 2$ ) nM for the C-33, C-63, and C-89 cases and 300 ( $\pm 30$ ) nM for the C-12 case (Figures 1 and 2). These dissociation constants correspond to free energies of association of  $-10.0$  kcal/mol for the C-33, C-63, and C-89 cases and  $-9.0$  kcal/mol for the C-12 case. A tighter association of C-33 relative to C-12 was also noted in a previous study utilizing TMRA labeled L7/L12 (Hamman et al., 1996a). The reason for this difference is not, at present, known, and we cannot be certain if it results from the intrinsic substitution of cysteine for

serine at residue 12 or arises as a consequence of the attachment of the fluorescence probes. A recent report by Bocharov et al. (1996), however, suggests that residue 12, in the N-terminal domain, is incorporated in an  $\alpha$ -helix, whereas residue 33 is not. This observation, coupled with the results from the TMRA and present studies, suggests that the integrity of the  $\alpha$ -helices is important for the subunit association.

The polarization data provide no evidence for tetramer formation of the full-length protein even at concentrations slightly higher than  $10^{-4}$  M. Also, no change in polarization was observed for C-89-F CTF from 200  $\mu$ M to 1 nM, indicating that the CTF remains monomeric over this concentration range, and demonstrating that significant interaction between C-terminal fragments does not occur in solution. Conversely, a dimer to monomer equilibrium for FITC labeled NTF, with a  $K_d$  near 300 ( $\pm 30$ ) nM, corresponding to a free energy of association of  $-9.0$  kcal/mol, was indicated by the concentration dependence of the polarization data. This result is consistent with the well-established role of the NTD in the dimerization of L7/L12 (Gudkov & Behlke, 1978; Gudkov et al., 1995). The data indicate that the dimerization of C-33, which corresponds to full-length L7/L12, is favored by 1.0 kcal/mol over that of the NTF, which differs from full-length L7/L12 only in that it lacks the C-terminal domains. These results suggest either that (1) labeling of the NTF with FITC disrupts the dimer association, possibly by interfering with one of the  $\alpha$ -helices as suggested above for the C-12 case, or (2) the C-terminal domains provide, either directly or indirectly, to full-length L7/L12 a free energy of stabilization corresponding to  $\sim 1.0$  kcal/mol. Should the latter case be correct, an indirect effect seems more likely than a direct interaction of the two C-terminal domains. An indirect effect would correspond, for example, to the C-terminal domains influencing another part of the protein, possible the hinge region. If a direct interaction of the C-terminal domains were present, even at the level of only 1.0 kcal/mol, one would expect to see more evidence of energy transfer between these domains since, given their effective high local concentration due to their tethering by the NTF, they would be in a dimeric state the majority of the time. This situation, i.e., two C-terminal domains tethered to an N-terminal fragment, may be compared to the case of well characterized tethered complexes such as FAD and flavinyltryptophan. In these molecular complexes, the two partners associate with free energies of approximately  $-1.0$  kcal/mol and exist as mixtures of closed forms  $\sim 90\%$  of the time and open forms the remaining time (Spencer & Weber, 1969; Visser et al., 1977). Time-resolved data on the mobilities of the C-terminal domains in full-length L7/L12 (Hamman et al., 1996c) also indicate that there is no significant direct interaction between these domains.

The absence of energy transfer in the case of the full-length C-terminal (C-63, C-89) labeled L7/L12 dimers (Table 2), suggests that these residues in the C-terminal domains are, on average, well-separated. The energy transfer observed with C-33-F and C-12-F, and also with C-33-A and C-12-A (Table 2), however, demonstrates that these intersubunit residues are in closer proximity in the dimeric N-terminal domain than are C-63 or C-89 in the C-terminal domains. Also, the fact that homo-FRET was observed in the double mutation variant, C-89:Δ42–52-F, which lacks

11 residues in the putative flexible hinge region, suggests that shortening this hinge pulls the two C-terminal domains closer together, a finding consistent with the formation of TMRA dimers in the C-89: $\Delta$ 42–52 variant (Hamman et al., 1996a). The assignment of the distance between the fluorophores in the N-terminal domain or in the C-terminal domain in the hinge-deleted double mutant, however, depends upon the orientation factor implied in eq 3. If one assumes  $r_{02}$  equal to zero (i.e.,  $\kappa^2$  equal to 2/3), one finds distances between C-33 residues or between C-12 residues in the range of 37–38 Å. These values seem large considering the previous demonstration that ground-state TMRA dimers can form in the TMRA labeled C-33 or C-12 variants (Hamman et al., 1996a) and the formation of zero-length cross-links (Oleinikov et al., 1993a). It may be that charge and/or steric constraints impose a difference between tetramethylrhodamine and fluorescein cases (one should note, for example, that fluorescein, though capable of ground-state dimer formation in solution, shows no evidence of such dimers in any of the L7/L12 variants so far investigated). One may also consider the problem from a different perspective. Specifically, to calculate  $r_{02}$ , which in effect gives the orientation between the appropriate dipoles which result in the observed polarizations, one can use eq 3 and assume that the fluorophores are close enough to experience complete transfer (i.e.,  $E = 1$ ). In the case of C-33, this calculation gives an angle of about 51°. Hence, the distances listed in Table 2 represent the distances assuming dynamic averaging, i.e.,  $\kappa^2$  equal to 2/3, but the actual distance between the fluorophores could be much less. The AEDANS data suggest that some self-transfer occurs between AEDANS moieties in the C-12 and C-33 positions even though the  $R_0$  is only  $\sim 11$  Å for AEDANS self-transfer. In the case of C-terminal domain fluorophores, which give either no evidence of energy transfer (C-63, C-89) or very little transfer (C-99), the distances listed in Table 2 are estimates based on the assumption of fast dynamic averaging ( $\kappa^2 = 2/3$ ), which is reasonable given the fact that the dynamic polarization results described in the previous paper indicate fast “local” probe motions and that the C-terminal domains have considerable mobility. These considerations suggest that the homo-FRET data presented here are not inconsistent with the TMRA data (Hamman et al., 1996a). Considering these results, a more appropriate description of L7/L12 would be in the form of a distribution of distances between the C-terminal domains and between the C-terminal and N-terminal domains. Such distance distributions can, in principle, be calculated from intensity decay data in the presence of energy transfer (Grinvald et al., 1972; Haas et al., 1975), and work along these lines is in progress.

The homo-FRET methodologies also allowed the demonstration of rapid and facile subunit exchange between populations of L7/L12 dimers at concentrations significantly above the dimer/monomer dissociation constants. The homo-FRET results were consistent with studies on the formation of TMRA ground-state dimers between labeled cysteine residues in the N-terminal domain and the subsequent rapid disappearance of these TMRA dimers upon addition of unlabeled L7/L12 (Hamman et al., 1996a). Weber (1992) has suggested that such subunit exchange occurs through cycles of dissociation, translational diffusion, and reassociation and, furthermore, that dissociation is the rate-limiting step in such a process. Experiments are in progress to

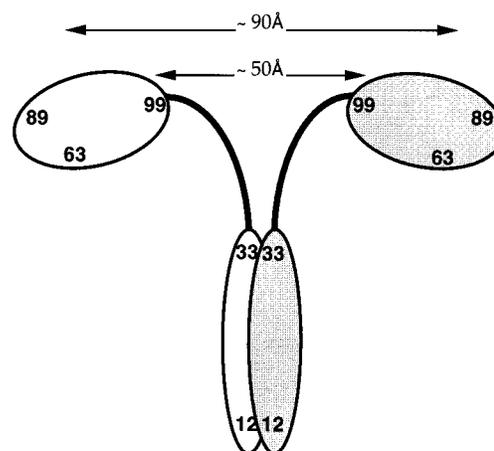


FIGURE 5: Schematic diagram indicating the flexible nature of L7/L12 and the likely average relative disposition of the C- and N-terminal domains.

determine the precise kinetics of the subunit exchange of free L7/L12 and also to determine if such exchange occurs between L7/L12 free in solution and bound to the ribosome.

The homo-FRET results indicate that the portions of the two C-terminal domains containing positions C-63 and C-89 are, on average, well-separated. The slight homo-FRET observed between the C-99 positions, however, suggests that the regions of the two C-terminal domains containing these residues, which are closer to the hinge region (based on the X-ray structure; Leijonmarck & Liljas, 1987) than C-63 or C-89, are closer to each other than are the C-63 or C-89 residues. These data are summarized in the schematic diagram presented in Figure 5. Our earlier work on the formation of ground-state TMRA dimers between N-terminal domain subunits but not between C-terminal domain subunits of L7/L12 (Hamman et al., 1996a) suggested that the C-terminal domains may be separated but did not provide any information on the degree of separation. The present work provides evidence for a significant average separation between the C-terminal domains which is consistent with the highly flexible nature of the protein discussed in the previous paper (Hamman et al., 1996c). This flexibility presumably facilitates the interaction of the two C-terminal domains of L7/L12 with other proteins on the ribosome which is presumed to be related to a functional role in factor binding (Dey et al., 1995).

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