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Solution Behavior of Proteins L7/L12 from the 50S Ribosomal Subunit of *Escherichia coli*†

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ABSTRACT: The behavior of *Escherichia coli* 50S ribosomal subunit proteins L7/L12 has been investigated in ribosome reconstitution buffer, TMK buffer, by sedimentation equilibrium and analytical gel filtration. Contrary to previous reports that L7/L12 exists in solution solely as dimer species [Möller, W., Groene, A., Terhorst, C., & Amons, R. (1972) *Eur. J. Biochem. 25*, 5], results presented here indicate that L7/L12 undergoes a monomer–dimer–tetramer self-association, with equal equilibrium constants of 3.5×10^4 M^{-1} obtained for the monomer–dimer and dimer–tetramer steps. These results yield standard Gibbs' free energies of -6.1 ± 0.6 kcal/mol at 20 °C. The observed absence of temperature dependence of this interaction over the range 5–25 °C indicates a zero standard enthalpy of self-association. Gel filtration results are presented that confirm the highly elongated shape of the L7/L12 molecule. The data suggest the corresponding Stokes radii for the monomer, dimer, and tetramer are 21–23, 26–28, and 29–32 Å, respectively. The significance of these results is discussed.

In previous studies of the 50S ribosomal subunit of *Escherichia coli*, the proteins L12 and its N-acetylated modification, L7, have been shown to dramatically influence the functional capability of the ribosome (Hamel et al., 1972; Weissbach et al., 1972; Highland et al., 1973, 1974; Möller, 1974; Koteliansky et al., 1977; Glick, 1977; Kurland, 1977). The absence of the proteins L7/L12 from the ribosome greatly reduces the capacity of the ribosome to support elongation factor dependent translation of synthetic poly(U) messages in vitro. Readdition of L7/L12 to the L7/L12-deficient ribosomes completely restores the activity lost by its removal (Hamel et al., 1972; Möller, 1974; Koteliansky et al., 1977; Glick, 1977). It has been shown that labeling of 50S subunits with antibody directed against proteins L7/L12 abolished the binding of elongation factor G to the 50S subunit, while labeling with antibodies directed against the other 32 proteins had no effect (Highland et al., 1974). The apparent involvement of L7/L12 in partial reactions involving initiation factors and release factors has been reviewed in detail (Möller, 1974).

L7/L12 are the only proteins present in multiple copies in the ribosome (Subramanian, 1975; Brimacombe et al., 1978). Though originally thought to be present in two copies per ribosome (Möller et al., 1972), the number of copies of L7

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plus L12 is currently believed to be four (Subramanian, 1975; Brimacombe et al., 1978; Pettersson, 1980). The number of copies each of L7 and L12 per ribosome evidently varies with growth stage of the cells from which the ribosomes are isolated (Subramanian, 1975). That the proteins L7/L12 may be located on the "surface" of the 50S subunit is indicated from immunoelectron microscopy (Tischendorf et al., 1975; Strycharz et al., 1978) and the reported ease with which they are selectively removed from the ribosome by treatment with 1 M NH_4Cl in the presence of 50% ethanol (Hamel et al., 1972).

Some physical properties of the proteins L7/L12 have been examined and reported. The entire 120 amino acid sequence has been determined, giving a molecular weight of 12 200 (Terhorst et al., 1973). The elongated shape of the molecule has been estimated from hydrodynamic studies (Wong & Paradies, 1972) and from low-angle X-ray scattering (Österberg et al., 1976). The tertiary structure has been predicted (Gudkov et al., 1977; Luer & Wong, 1979) from sequence and circular dichroism analyses. It has been generally accepted that L7/L12 exists in ordinary buffers as a dimer. This latter conclusion was derived from the first reported characterization of L7/L12 by sedimentation equilibrium. Those studies provided molecular weight estimates in the range 19 000–23 000 (Möller et al., 1972) with seemingly linear ln C vs. r^2 plots. However, the observation of an L7/L12 species exceeding a molecular weight of 40 000 daltons (Möller et al., 1972) was also reported.

These results and the somewhat variable and low estimate of a dimeric molecular weight suggest that the accepted definition of the solution quaternary structure be tenuous. Examples of such systems yielding linear ln C vs. r^2 plots have been described (Aune, 1978).

From the preceding discussion, it is evident that, in order to study interactions between proteins L7/L12 and any other ribosomal constituent, a more detailed analysis of the solution behavior of proteins L7/L12 is necessary. The characterization of the solution behavior of proteins L7/L12 has been undertaken by a combination of analytical gel filtration and sedimentation equilibrium techniques. The sedimentation equilibrium technique of studying protein–protein interactions is firmly founded on thermodynamic principles since both chemical and mechanical equilibria are established at sedimentation equilibrium. The application of analytical gel filtration methodology to the study of protein–protein interactions has also been discussed in detail (Ackers, 1970, 1975). This communication presents a thermodynamic characterization of the tendency of L7/L12 to self-associate in ribosome reconstitution buffer.

Materials and Methods

All chemicals used in this study were standard reagent grade. Urea was purchased from Fisher Scientific. Concentrated solutions of urea were treated with 5 g of activated charcoal per L of solution, successively filtered through Whatman No. 1 paper and Millipore type HA membrane, and then treated with 10 g of Bio-Rad AG-501-X8 mixed-bed ion-exchange resin and refiltered through a Millipore type HA membrane. The conductance of the urea concentrates used was less than 5 $\mu\Omega^{-1}$. Guanidine hydrochloride (Gdn·HCl)[1] was purchased from Bethesda Research Laboratories. Phosphocellulose was purchased from Schwarz/Mann (0.82 mequiv/g); DEAE-cellulose, CM-cellulose, and $NaDodSO_4$ were purchased from Bio-Rad Laboratories. All other solutions used in this study were filtered through Millipore type HA membrane. All dialyses were performed with Spectrapor tubing having molecular weight cutoffs not exceeding 3500.

Protein Purification. Ribosomes (70 S) were isolated from *Escherichia coli* strains B and MRE-600 cells. The 50S subunits were separated from 30S subunits by sucrose gradient zonal centrifugation at low Mg^{2+} concentrations as previously described (Rohde et al., 1975). Proteins were obtained from the 50S subunits by extraction in 67% acetic acid (Zimmermann & Stöffler, 1976). The proteins L7/L12 were purified from the 67% acetic acid extract by chromatography on phosphocellulose in 7.5 M urea followed by rechromatography on DEAE-cellulose in 7.5 M urea (Zimmermann & Stöffler, 1976). The L7/L12-containing fractions from the DEAE-cellulose effluent were subjected to a second rechromatography step on CM-cellulose in 7.5 M urea and 0.04 M sodium acetate, pH 5.6. The resultant L7/L12-containing fractions were pooled, dialyzed to 15% acetic acid, concentrated by lyophylization, redissolved in 7.0 M urea, 0.05 M sodium phosphate, 0.2 M sodium chloride, and 1% 2-mercaptoethanol, pH 7.0, and incubated at room temperature for 30 min. This solution was then subjected to a final Sephacryl S-300 chromatographic step on a 1.5 × 95 cm column in 7.5 M urea, 0.05 M sodium phosphate, 0.2 M sodium chloride, and 10 mM 2-mercaptoethanol, pH 5.8. L7/L12 so prepared was stored frozen in 15% acetic acid for further use.

Polyacrylamide Gel Electrophoresis. Electrophoresis was routinely performed in 10-cm 9.0% acrylamide–0.5% bis(acrylamide) gels containing 8 M urea at pH 4.5. The gels were run at a constant current of 3 mA/gel at 5–8 °C.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed in 12.5% acrylamide gels with 1.25% bis(acrylamide) as previously described (Weber & Osborn, 1969; Prakash & Aune, 1978). Egg white lysozyme (M_r 14 300), pancreatic ribonuclease A (13 800), α-chymotrypsin (11 000; 13 000), whale myoglobin (17 200), soybean trypsin inhibitor (21 600), carbonic anhydrase (30 000), and pepsinogen (40 400) were routinely employed as standards.

Amino Acid Composition. The amino acid compositions of proteins L7/L12 were determined from 24-h acid hydrolysates of 50–100-μg samples as described by Rohde et al. (1975). The amino acid compositions so obtained were compared with those reported by other investigators (Kaltschmidt et al., 1970; Mora et al., 1971; Terhorst et al., 1973) by methods previously discussed (Rohde et al., 1975; Prakash & Aune, 1978).

Protein Refolding. Purified samples of proteins L7/L12 in 15% acetic acid were lyophylized to dryness, then dissolved in 50–100 μL of 6 M urea, 1% 2-mercaptoethanol, and 0.15 M Tris-HCl, pH 8.0, and incubated at 25 °C for 20 min. Following incubation, 9 volumes of TMK buffer, pH 7.5, were added with gentle mixing. The resulting solution was dialyzed against six to eight changes of 20 volumes of TMK buffer, pH 7.5, in the cold, allowing 18–24 h for final dialysis equilibrium.

Specific Absorptivities. Ultraviolet absorptivities of proteins L7/L12 in TMK buffer pH 7.5, were determined by methods previously described (Aune & Timasheff, 1971; Rohde et al., 1975; Prakash & Aune, 1978) by using egg white lysozyme as a reference standard (Aune & Tanford, 1969).

Partial Specific Volumes. The partial specific volume of L7/L12 in TMK buffer, pH 7.5, was calculated from its amino acid composition (Cohn & Edsall, 1943). The partial specific

[1] Abbreviations used: DEAE, diethylaminoethyl; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; TMK buffer, buffer solution containing 0.03 M Tris, 0.02 M $MgCl_2$, and 0.35 M KCl, pH 7.4; Gdn·HCl, guanidine hydrochloride; DMSI, dimethylsuberimidate; Tris, tris(hydroxymethyl)aminomethane.

volume in 6 M Gdn·HCl was calculated by the method of Lee & Timasheff (1974). The partial specific volumes so estimated for L7/L12 are 0.755 in both TMK buffer, pH 7.4, and 6 M Gdn·HCl.

Sedimentation Equilibrium. High-speed sedimentation equilibrium experiments (Yphantis, 1964) as modified (Kar & Aune, 1974) were conducted with solutions containing 0.05–0.5 mg/mL protein in TMK buffer, pH 7.4, or unbuffered 6 M Gdn·HCl at 5–25 °C. The experiments were performed in two Beckman Model E analytical ultracentrifuges, one of which was equipped with a photoelectric scanner, by methods previously described (Kar & Aune, 1974; Rohde et al., 1975; Aune & Rohde, 1977).

Analytical Gel Chromatography. Elution gel chromatography was performed at room temperature (23–25 °C). L7/L12 (1–2 mg) in 250 μL of TMK buffer, pH 7.4, was applied to a 0.9 × 50 cm Sephadex G-100 superfine column, equilibrated with TMK buffer, pH 7.4, and allowed to run into the gel by gravity flow. The sample was washed into the column with 500 μL of TMK buffer, pH 7.4, and then eluted with TMK buffer, pH 7.4, by gravity flow under a hydrostatic pressure head of 90 cm. Fractions were collected by weight from the time of sample application. Elution was monitored by ultraviolet absorbance at 230 nm in a Zeiss PM2 spectrophotometer with quartz cells of 1-cm path length. Partition coefficients were determined as described previously (Prakash & Aune, 1978) by using fractionated blue dextran 2000 and DNP-glycine as void and included volume markers, respectively. Stokes' radii were calculated from the partition coefficients by the procedure of Ackers (Ackers, 1970) using eq 1:

$$R_s = B + A \operatorname{erfc}^{-1}(\sigma) \quad (1)$$

where σ is the experimentally determined partition coefficient, R_s is the Stokes' radius, and A and B are constants. Ovalbumin, bovine carbonic anhydrase, trypsin inhibitor, myoglobin, and cytochrome *c* were employed as calibration standards.

Chemical Modification. (*i*) *Dimethylsuberimidate Cross-Linking.* L7/L12 was cross-linked by a modification of the procedure of Davies & Stark (1970); 1–2 mg of L7/L12 was dissolved in 250 μL of 6 M Gdn·HCl and dialyzed against eight 40-volume changes of 0.2 M Tris-HCl, pH 8.5, containing 1.5 mg of dimethylsuberimidate. The mixture was allowed to stand at room temperature (23–25 °C) for 2 h and then dialyzed against six 40-volume changes of TMK buffer, pH 7.4, at 4 °C.

(*ii*) *Hydrogen Peroxide Oxidation.* Hydrogen peroxide oxidation of L7/L12 was performed according to the procedure of Neumann (1967). To a 500-μL solution containing 1–2 mg of L7/L12 in 0.02 M Tris-$HClO_4$ was added 1 N $HClO_4$ to bring the pH to 2.2. The solution was brought to 30 °C in a temperature block, 30 μL of 30% H_2O_2 was added, and the solution was incubated for 2 h at 30 °C. The reaction mixture was then dialyzed against four 20-volume changes of TMK buffer, pH 7.4, at 4 °C.

Protein Concentration Determinations. Occasionally protein concentrations were checked by the colorimetric method of Lowry et al. (1951) using egg white lysozyme as the calibration standard. Volumes of protein-containing solutions were adjusted to 200 μL with distilled water to give a total reaction volume of 1.3 mL. Color development was quantitated by the absorbance of the solution at 750 nm.

Results

Protein Identification and Purity. The final identification of and estimate of purity of the L7/L12 preparations used in this study have been based on comparisons of the amino acid compositions of three preparations with those previously reported (Kaltschmidt et al., 1970; Mora et al., 1971), the electrophoretic mobility and appearance of these proteins on $NaDodSO_4$ polyacrylamide gels and on 8 M urea (pH 4.5)–polyacrylamide gels (Zimmermann & Stöffler, 1976), sedimentation equilibrium concentration distributions in 6 M Gdn·HCl, and the position of elution of these proteins from phosphocellulose, DEAE-cellulose, CM-cellulose, and Sephacryl S-300 columns (Zimmermann & Stöffler, 1976).

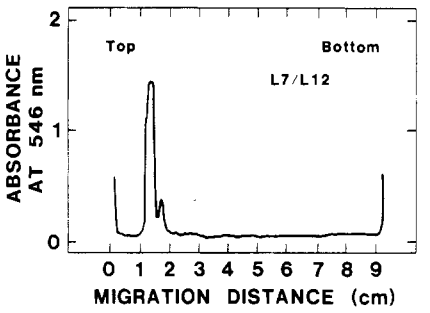

FIGURE 1: Absorbance scan of Coomassie blue stained acid–urea electrophoresis gels of L7/L12 at 546 nm. Gel composition is 8 M urea, 9% acrylamide, and 0.5% bis(acrylamide). Gel was run at pH 4.5.

The amino acid compositions of the L7/L12 preparations used in this study when compared with those of Dzionara et al. (1970) and Mora et al. (1971) and that derived from the amino acid sequence (Terhorst et al., 1973) gave correlation coefficients of 0.990, 0.994, and 0.993, respectively. Comparisons of the amino acid compositions of the L7/L12 preparation used in this study with amino acid compositions reported in the literature for the other 50S subunit proteins yielded correlation coefficients ranging from 0.72 to 0.94. The amino acid composition derived from the L7/L12 sequence (Terhorst et al., 1973) gave similar results when subjected to these tests. This means that the protein preparations used in this study are the same as those reported as L7/L12 by other investigators.

Figure 1 shows a representative scan of the 8 M urea–polyacrylamide gel electrophoresis (pH 4.5) of approximately 20 μg of L7/L12. It is evident that these L7/L12 preparations consist of approximately 90% L7 and 10% L12, assuming both L7 and L12 have the same affinities for Coomassie blue stain. There is no evidence of contamination by other ribosomal proteins in these preparations. Electrophoresis of these L7/L12 preparations on $NaDodSO_4$–polyacrylamide gels at loads of 10–20 μg produced single bands with molecular weight estimates of 13 500. This compares quite favorably with the $NaDodSO_4$ molecular weight estimates reported in the literature (Dzionara et al., 1970). Table I summarizes the molecular weight estimates obtained for L7/L12 from three separate preparations.

Table I also shows the molecular weight estimated from two sedimentation equilibrium experiments in 6 M Gdn·HCl at 18 °C. The $\ln f$ vs. r^2 plots (f is fringe displacement) were entirely linear. More importantly, the curve fitting of the radial concentration distributions as described by Aune & Rohde (1977) and Rohde et al. (1975) gave the same results. Hence, in 6 M Gdn·HCl, L7/L12 is unambiguously monodisperse. Consequently, the fringe displacement data are not shown for these experiments. The molecular weight for L7/L12 determined in 6 M Gdn·HCl compares quite favorably with that estimated from $NaDodSO_4$ electrophoresis as well as that derived from the amino acid sequence (Terhorst et al.,

Table I: Summary of Some Physical Properties of 50S Ribosomal Protein L7/L12

property	
molecular weight	
sedimentation equilibrium[a]	
TMK buffer	heterodisperse
6 M Gdn·HCl	12 000
$NaDodSO_4$-polyacrylamide gel electrophoresis[a]	13 500
amino acid sequence[b]	12 200
partial specific volumes	
$\bar{\nu}$	0.755[c]
ϕ'_2	0.755[d]
specific absorptivity[a]	0.06 mL/(mg·cm) at 275 nm
frictional ratio (f/f_0)	1.6[e]

[a] Determined in this work. [b] Terhorst et al. (1973). [c] Calculated by method of Cohn & Edsall (1943). [d] Calculated by method of Lee & Timasheff (1974). [e] Determined from gel chromatography.

1973). The sedimentation equilibrium results, although less stringent in defining trace contamination than electrophoretic methods, provide assurance that the L7/L12 preparations used here are at least 95% homogeneous under the conditions employed.

Absorptivity of L7/L12. The ultraviolet absorptivity of L7/L12 was estimated with refolded preparations of this protein in TMK buffer, pH 7.4, at protein concentrations of 2–4 mg/mL by methods previously discussed (Aune & Timasheff, 1971; Rohde et al., 1975). With these procedures, the absorptivity of L7/L12 was estimated to be 0.06 mL/(mg·cm) at 275 nm and is also listed in Table I.

Sedimentation Equilibrium of L7/L12 in TMK Buffer Direct from Refolding. Purified preparations of L7/L12 were refolded from 6 M Gdn·HCl as described under Materials and Methods and subjected to sedimentation equilibrium in TMK buffer, pH 7.4. Experiments were performed at initial protein concentrations of 0.15–0.45 mg/mL and at temperatures in the range 5–25 °C. Initial protein concentrations were estimated from ultraviolet spectra (230–240-nm region) of the refolded concentrated protein solutions from which the sedimentation equilibrium samples were prepared by dilution and occasionally checked in the final sample by the Lowry method (Lowry et al., 1951) as described above. In each case, the initial protein concentration was inferred by numerical integration of the concentration distribution obtained from the interference optics. These concentrations were always in agreement with concentrations determined by the method of Lowry and by absorptivities when applicable.

Figure 2 shows the data from a representative sedimentation equilibrium experiment at 18 °C for L7/L12 direct from refolding; the inset shows the same data replotted in the ln f vs. r^2 format. The ln f vs. r^2 representation gives a very linear plot, yielding a molecular weight of 20 100. This value, which is identical with the values reported for L7/L12 in previous studies (Möller et al., 1972), is obviously too large to represent monomeric species and too small to represent homogeneous dimeric species since the ultimate molecular weight determined in this study is 12 200. $NaDodSO_4$ electrophoresis of the samples centrifuged both prior to and following centrifugation have shown the presence of only one band of apparent molecular weight 13 500.

Since it is possible for self-associating systems to yield seemingly linear ln f vs. r^2 plots (Aune, 1978), the data of Figure 2 were fit directly to a variety of self-association models. Only two models could be found which completely describe

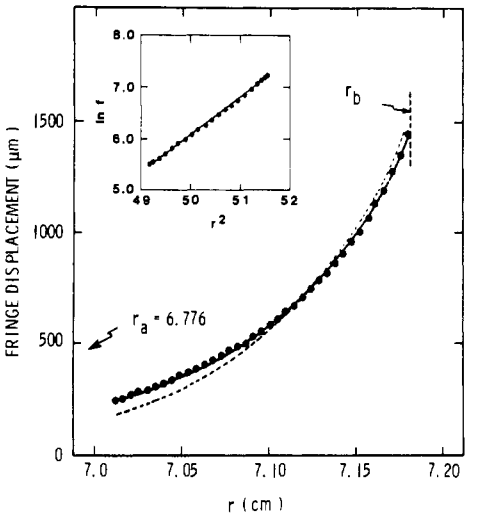

FIGURE 2: Sedimentation equilibrium of L7/L12 in TMK buffer, pH 7.5. Sedimentation equilibrium was performed at 25 980 rpm, 18 °C, with an initial protein concentration of 0.4 mg/mL and a sample volume of 0.15 mL. The solid line is the best fit of the data, assuming a monomer–dimer–tetramer mixture. The dashed line is the best fit of the data assuming only dimers present. (Inset) ln f vs. r^2 replot of the same data. The solid line is the linear least-squares fit of the data, yielding a molecular weight of 20 100.

these data, a monomer–dimer–tetramer model and a monomer–dimer–trimer–tetramer model. As shown by the solid line in Figure 2, both of these models accurately describe the entire fringe distribution of the experiment with average residuals on the other of 3–5 μm, well within the normal limits of plate-reading error. The dashed line in Figure 2 represents the best fit obtained for these data assuming that only dimers are present in the sample. This illustrates the invalid conclusion derived from the inset figure. Although this model appears to describe the data at high fringe displacements fairly well, it fails at the lower fringe displacements where residuals reach 50 μm at the low concentration extreme. Monomer–dimer models produced acceptable fits from the low through the moderate fringe displacements but failed at the high fringe displacements. Dimer–tetramer models produced exactly the same results as that assuming only dimers were present. Finally, monomer–dimer–trimer models fit most of the data quite well but deviate significantly from the three to four data points closest to the base of the cell with deviations reaching 40 μm at the last datum collected.

More than 20 individual sedimentation equilibrium experiments have been conducted in TMK buffer, pH 7.4, with L7/L12. In some cases the data obtained can be fit to an indefinite self-association model, while in others the data can be fit to a monomer–dimer–trimer model in addition to the models presented above. However, only the monomer–dimer–tetramer model always produces fits of the data collected to within normal plate-reading error. For this reason, the monomer–dimer–tetramer model has been adopted for the analysis of L7/L12 sedimentation data in TMK buffer, pH 7.4, as shown in eq 2 and 3.

$$2(L7/L12) \overset{K_{12}}{\rightleftharpoons} (L7/L12)_2 \quad (2)$$

$$2(L7/L12)_2 \overset{K_{24}}{\rightleftharpoons} (L7/L12)_4 \quad (3)$$

It is to be noted that no species of L7/L12 larger than tetramers have ever been observed in sedimentation equilibrium experiments in TMK buffer, pH 7.4, at fringe displacements up to 1800 μm. Therefore, it is evident that L7/L12 exists in TMK buffer, pH 7.4, as a mixture of species, monomers, dimers, and tetramers.

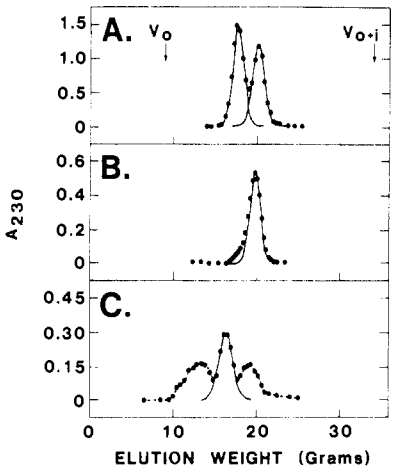

FIGURE 3: Analytical gel chromatography of L7/L12 derivatives on Sephadex G-100 superfine in TMK buffer, pH 7.4, at 25 °C. Column size is 0.9 × 50 cm. Initial sample volume is 0.5 mL. V_o is the elution position of blue dextran and V_{i+o} is the elution position of DNP-glycine. (A) Refolded L7/L12 at initial concentration of 4.2 mg/mL. Elution position of this material is concentration dependent (see text). (B) Refolded L7/L12 with methionine residues oxidized as described under Materials and Methods. Initial concentration is 1.2 mg/mL. (C) DMSI cross-linked refolded L7/L12. Initial protein concentration is 1.2 mg/mL. The solid lines are the best Gaussian reconstructions of the eluted peaks.

Sedimentation Equilibrium Analysis of Sephadex G-100 Fractionated Refolded L7/L12. L7/L12 (1–1.5 mg) was refolded from 250 μL of 6 M Gdn·HCl by dialysis against eight 40-volume changes of TMK buffer, pH 7.4, in the cold and then chromatographed on Sephadex G-100 superfine as previously described. As shown in Figure 3A, the elution profile illustrates two peaks. The fractions at the maximum of each peak were dialyzed against one 10-mL change of TMK buffer, pH 7.4, in the cold for 24 h and subjected to a sedimentation equilibrium experiment. Each experiment consisted of a program of at least two speeds at 20 °C.

The material present in the peak having the smaller partition coefficient yielded the usual monomer–dimer–tetramer mixture of species. However, the proportion of species larger than monomer was greater in this sample than that derived from unfractionated L7/L12. The material from the peak having the larger partition coefficient consistently demonstrated the presence of monomer and a small amount of dimer. The latter peak composition was 90–95% monomeric species and 10–5% dimeric species.

This doublet elution profile of L7/L12 has been observed in each of six separate experiments. In each case, the peaks were separated by 2–2.5 g of elution weight, equivalent to a partition coefficient difference of 0.1–0.15. Furthermore, as shown in Figure 4, the partition coefficients of both peaks exhibited a marked concentration dependence. As the concentration of L7/L12 applied to the column (hence, the average running concentration) increased, a corresponding decrease in the apparent partition coefficient of each peak was observed. The decrease in partition coefficient of each peak was such that the peaks always appeared to be coupled in the effluent.

The consistent appearance of doublet peaks in the elution profile of L7/L12 of Sephadex G-100 superfine reflects the existence of two populations of L7/L12 having different capabilities for self-interaction in the refolded material. Sedimentation equilibrium analysis of the material in the peaks having the lower partition coefficient of the doublet consistently yields distributions of monomeric, dimeric, and tetrameric species compatible with a monomer–dimer–tetramer model

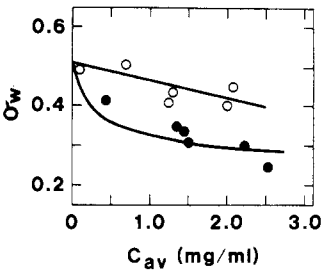

FIGURE 4: Concentration dependence of the weight-average partition coefficients, σ_w, obtained for refolded L7/L12 on Sephadex G-100 superfine in TMK buffer, pH 7.4, at 25 °C. Data obtained as in Figure 3A. C_{av} is the average running protein concentration in the column. Filled circles represent data for peaks of smaller σ_w, and open circles represent data for peaks as in Figure 3A. The solid lines are theoretical curves relating weight-average partition coefficients of each species, σ_i, and the weight fraction of each species, f_i, present in the eluted material (see text for discussion).

in equilibrium with equal equilibrium constants of 3.5×10^4 M^{-1} for each of the two steps involved. The material in the peaks having the larger partition coefficient in the doublet when treated as a self-interactive system by sedimentation equilibrium analysis revealed the distribution of monomeric and dimeric species present. The data were consistent with the material in the peak having an interaction constant of 9.0×10^2 M^{-1}. This interaction constant is small enough that only monomers and dimers will be observed in sedimentation equilibrium experiments at all protein concentrations less than 5 mg/mL, a concentration far greater than either the maximum concentration generated in the centrifuge or the maximum average running concentration achieved in the gel chromatography experiments. Although it might be argued that this material could represent a mixture of material as capable of self-interaction as that obtained from the peak having the smaller partition coefficient and material having no capability for self-interaction (incompetent), this is inconsistent with both the observed concentration dependence of the material in this peak as well as the coupling of this peak to the elution position of the peak having the lower partition coefficient. If this material represented a truly incompetent species, then 30% of the total L7/L12 in the original sample should chromatograph as monomeric material with an essentially invariant partition coefficient at the initial column loading concentrations employed since the concentration dependence factor for proteins is less than 0.01 (Ackers, 1970). Therefore, it is concluded that the material in each peak is competent to interact, but that the material in the peak having the smaller partition coefficient is about 40 times more competent than the material in its companion peak and that the coupling of the two peaks is reflected as the average of the two interaction constants (approximately 2×10^4 M^{-1}).

Table II summarizes the equilibrium constants calculated from the sedimentation equilibrium analyses of the material from the peaks having the smaller partition coefficient and those calculated from sedimentation equilibrium analyses of unfractionated L7/L12. It is clearly evident that the Sephadex G-100 fractionated material yields association constants approximately 2-fold larger than those derived from the unfractionated material. Approximately 60% of the total material applied to the gel chromatography column elutes in the peak having the smaller partition coefficient, and 40% elutes in the companion peak having the larger partition coefficient. When the unfractionated L7/L12 subjected to sedimentation equilibrium analysis is corrected for this distribution of total material, the association constants obtained are equivalent to those obtained from the fractionated L7/L12. It is noted here

Table II: Summary of Self-Association Equilibrium Constants of Protein L7/L12 Determined by Sedimentation Equilibrium in TMK Buffer

preparation method	K^a (10^{-4} M)	K^a (10^{-4} M)
unfractionated[b]	2.1	1.5
fractionated, no Mg^{2+} [c]	1.5	1.3
G-100 fractionated[d]		
low σ	4.1	3.2
high σ	0.09	0.09

[a] Equilibrium constants as defined by eq 2 and 3. [b] Unfractionated material was subjected to sedimentation equilibrium direct from refolding. [c] $MgCl_2$ was omitted from the usual TMK buffer. [d] Refolded L7/L12 was chromatographed on Sephadex G-100 superfine prior to conducting sedimentation equilibrium analysis. High ahd low σ refer to the particular eluted peak from which material was derived for analysis.

that the equilibrium constants calculated for L7/L12 from experiments at different angular velocities are all within two standard deviations from the mean values presented in Table II.

Since 40% of the total L7/L12 chromatographed on Sephadex G-100 was found to be very weakly competent to self-interact, it was of interest to determine whether this fraction of material could be induced to assume a more self-interactive form. It has been recently reported from studies of thermal changes in the circular dichroic spectrum that L7/L12 undergoes a maximum degree of unfolding upon heating to 90 °C and that its original folded state is completely restored with slow cooling to room temperature (Luer & Wong, 1980). If the fraction of refolded L7/L12 showing only a weak tendency to self-associate was reversibly trapped in a thermodynamic state distinct from that showing the greater tendency for self-association, it might be possible to reversibly heat denature this material and then renature it to full self-association capability. If this weak tendency to self-associate is the result of some irreversible change of the protein, then the same sedimentation equilibrium results should be obtained following a reversible thermal denaturation–renaturation program as were obtained before it.

Such a thermal denaturation–renaturation experiment was performed with the L7/L12 fractions having the larger partition coefficients from two individual Sephadex G-100 chromatographic experiments. L7/L12 in TMK buffer, pH 7.4, was heated in a 5-L circulating water bath to 90 °C for a 4-h period and then allowed to cool to room temperature in 10 h. Sedimentation equilibrium experiments were then performed on each of the samples for two different speeds each. Curve-fitting analysis of these data yielded the presence of 90–95% monomeric species and 10–5% dimeric species for each of the two experiments. These results are the same as those obtained prior to thermal denaturation–renaturation and are consistent with the association constant of 9.0×10^2 M^{-1} previously discussed. Therefore, it appears that this material represents an irreversibly modified fraction of L7/L12. The nature of this modification will be discussed later in the text.

It is evident from the concentration dependence of the data in Figure 4 that the partition coefficients determined for refolded L7/L12 are actually weight-average partition coefficients, σ_w. By use of the association constants determined from sedimentation equilibrium experiments and the average running concentrations for the material in each chromatographic peak, the weight fraction, f_i, of each species present in each of the peaks may be calculated. Then the partition coefficients of each species, σ_i, may be estimated from the resulting system of equations defined by (Ackers, 1970)

$$\sigma_w = \sum_i f_i \sigma_i \quad (4)$$

When the matrices defined by this system of equations for all chromatographic runs are solved to within an experimental error $\Delta\sigma_w \simeq 0.04$, the partition coefficients of each species are found to be $\sigma_1 = 0.51$ ($R_s = 22$ Å), $\sigma_2 = 0.29$ ($R_s = 27$ Å), and $\sigma_4 = 0.23$ ($R_s = 29$ Å).

The solid lines through the data in Figure 4 were generated from these partition coefficients and the relations for the weight fraction of each species in a monomer–dimer–tetramer equilibrium:

$$f_1 = C_1/C_{av} \quad (5)$$

$$f_2 = 2KC_1^2/(M_1C_{av}) \quad (6)$$

$$f_4 = 4K^3C_1^4/(M_1^3C_{av}) \quad (7)$$

where C's are weight concentrations of the indicated species, C_{av} is the average running concentration of total protein in the appropriate chromatographic peak, M_1 is the monomer molecular weight, and K is the association constant for each step in the self-association equilibrium (unsubscripted here since both steps in the monomer–dimer–tetramer equilibrium have the same value for the equilibrium constant).

Sephadex G-100 Chromatography of L7/L12 Derivatives. In an effort to fix the partition coefficients of the various L7/L12 species, two types of chemically modified L7/L12 were subjected to gel chromatography in TMK buffer, pH 7.4, with subsequent sedimentation equilibrium analysis. L7/L12 was oxidized by hydrogen peroxide to obtain completely monomeric protein for chromatographic analysis (Koteliansky et al., 1978) and was cross-linked with dimethylsuberimidate to obtain chemically stable dimeric and tetrameric species (Koteliansky et al., 1978).

Figure 3B shows a representative elution profile for the oxidized L7/L12. Although the leading edge of the profile is somewhat skewed from true Gaussian shape, only monomeric species were detected by sedimentation equilibrium analysis at fringe displacements up to 1000 μm. Two independent refoldings of L7/L12 have been treated with hydrogen peroxide and chromatographed on Sephadex G-100 superfine. Each time the material eluted as a single peak with the same partition coefficient, and when the eluted peak was rechromatographed, the elution position was unchanged. The average partition coefficient for oxidized L7/L12 is 0.45 ± 0.03, corresponding to a Stokes' radius of 24 Å. Although sedimentation equilibrium analysis clearly shows this material to be monomeric, the Stokes' radius obtained is approximately 2 Å larger than that inferred for monomeric unmodified L7/L12. This suggests that not only is oxidized L7/L12 incapable of self-interaction but also it is less compact than the unmodified protein.

Figure 3C shows the elution profile obtained with dimethylsuberimidate cross-linked L7/L12. The three peaks obtained have partition coefficients of 0.17_5, 0.29_0, and 0.40_5. The shape of the three peaks obtained deviate significantly from Gaussian, indicating that each may be the result of a complex mixture of species.

The fractions from each of the three dimethylsuberimidate cross-linked L7/L12 peaks were concentrated 2–4-fold by dry dialysis against Aquacide IIA, dialyzed 24 h against one 10-mL change of TMK buffer, pH 7.4, and then subjected to sedimentation equilibrium analysis. The results of the curve-fitting analysis for each of these samples are listed in Table III. As indicated in the table, the three peaks that eluted from the gel filtration column are heterodisperse. The pooled peak fraction corresponding to partition coefficient 0.40_5

Table III: Summary of Sedimentation Equilibrium Analysis of L7/L12 Derivatives

chemical derivative[a]	σ[b]	composition[c] (%) monomer	dimer	tetramer
Met oxidized	0.45	100		
DMSI cross-linked	0.41	70	30	
	0.29	54	32	14
	0.18		19	81

[a] As described under Materials and Methods. [b] Partition coefficient of material on Sephadex G-100 superfine in TMK buffer. [c] Determined by sedimentation equilibrium analysis.

at sedimentation equilibrium was resolved as a mixture of 70% monomer and 30% dimer, the pooled peak fraction corresponding to partition coefficient 0.29_0 at sedimentation equilibrium was resolved as a mixture of 54% monomer, 32% dimer, and 14% tetramer, and the skewed peak corresponding to partition 0.17_5 was distributed as a mixture of 19% dimer and 81% tetramer.

Since the fraction of each species present in the material comprising each of the three DMSI peaks is known from sedimentation equilibrium analysis, an estimate may be made of the partition coefficients of each of the three DMSI species from the weight-average partition coefficients determined for each peak. Such an analysis yields $\sigma_{1X} = 0.48$ ($R_s = 23$ Å), $\sigma_{2X} = 0.24$ ($R_s = 28$ Å), and $\sigma_{4X} = 0.16$ ($R_s = 31$ Å). (The additional subscript "x" is used here to denote cross-linked material.)

Effect of Mg^{2+} on the Self-Association of L7/L12. Since it has been alluded to previously that the molecular weight of L7/L12 might depend on the presence or absence of Mg^{2+} in the protein solution (Wong & Paradies, 1974), three sedimentation equilibrium experiments were performed on L7/L12 refolded in the absence of Mg^{2+}. The concentration distributions obtained were curve fit as previously described, and equilibrium constants were calculated as before. The results of these experiments are summarized in Table II. Since the equilibrium constants calculated for these experiments are the same as those obtained for L7/L12 in the presence of 20 mM Mg^{2+}, it must be concluded that Mg^{2+} has no effect on the L7/L12 self-association in the concentration range supporting maximal ribosome activity in protein synthetic reactions.

Thermodynamic Parameters of L7/L12 Self-Association. The standard Gibbs' free energy, $\Delta G°$, for the self-association of L7/L12 can be calculated from the equilibrium constants discussed above by use of eq 8

$$\Delta G° = -RT \ln K \quad (8)$$

where K is the association constant for one step in the equilibrium model represented by eq 2 and 3. The temperature dependence of the equilibrium constant can be analyzed in the usual fashion to yield a van't Hoff $\Delta H°$ for association.

Sedimentation equilibrium experiments were conducted with refolded L7/L12 over the temperature range 5–25 °C. Equilbrium constants calculated for each of the two steps in the proposed model above were the same at all temperatures studied, i.e., $K_{12} = K_{24} = 3.5 \times 10^4$ M^{-1}. The absence of a significant temperature effect is consistent with a zero van't Hoff enthalpy of association. The corresponding thermodynamic parameters of the L7/L12 self-association are $\Delta G° = -6.1$ kcal/mol, $\Delta H° = 0.0$ kcal/mol, and $\Delta S° = 21.0$ eu.

Although the type of interaction involved in the self-association of L7/L12 cannot be deduced from these data, they do not appear to be consistent with a primarily hydrophobic interaction. Hydrophobic interactions are usually characterized by a temperature-dependent enthalpy that may be small near 0 °C and increases with temperature. Electrostatic interactions certainly play a significant role in the self-association of L7/L12, for as the pH of the solution is lowered to the isoelectric point of the protein, the degree of association increases to the point where molecular weight averages of nearly 200 000 are obtained by sedimentation equilibrium. As the pH is further reduced to pH 2.2, the molecular weight averages decrease somewhat, but still exceed 100 000.

Discussion

Several interesting but previously unreported properties of L7/L12 have been observed which warrant discussion. First, the ultraviolet absorptivity of L7/L12 in the 270–280-nm region is remarkably low. This is the most obvious consequence of the existence of only two aromatic residues, phenylalanine, in the entire 120 amino acid residue sequence (Terhorst et al., 1973). The only remarkable feature of the L7/L12 spectrum is the characteristic fine structure triplet of phenylalanine red shifted about 5 nm from the phenylalanine maximum of 259 nm. In TMK reconstitution buffer, the absorbance of moderately concentrated L7/L12 solutions becomes reasonably strong only at wavelengths below 240 nm. Although it is difficult to obtain accurate estimates of L7/L12 concentration spectrophotometrically, utilizing refractometrically calibrated absorbances at 230–236 nm have provided reasonable concentration estimates, equivalent to those obtained from Lowry determinations.

The ultimate molecular weight estimates of L7/L12 preparations used in this study are internally self-consistent and consistent with all previously reported values. The values of 13 500 ± 1500 obtained from $NaDodSO_4$–polyacrylamide gels and 12 000 ± 400 from sedimentation equilibrium in 6 M Gdn·HCl are identical within the experimental error. These molecular weight estimates compare quite favorably with previously reported values (Dzionara et al., 1970; Mora et al., 1971; Möller et al., 1972; Brimacombe et al., 1978) and are identical with that obtained from the amino acid sequence (Terhorst et al., 1973), within experimental error.

A major difference has been observed in the behavior of L7/L12 in TMK reconstitution buffer from that previously suggested in the biochemical literature. It has been repeatedly stated that L7/L12 exists entirely as dimers of molecular weight 24 000 in TMK buffer (Wong & Paradies, 1974; Österberg et al., 1976; Gudkov et al., 1977). The results reported here are not entirely consistent with this view. Although 25–30% of the total L7/L12 is present as dimers in TMK buffer, these experiments consistently indicate the presence of 30–55% monomers and 15–30% tetramers as well, depending on protein concentration. This tendency has been observed at several angular velocities in the ultracentrifuge over a temperature range of 5 to 25 °C. In no case has any of these data been consistent with the presence of only dimeric species, although apparently linear $\ln f$ vs. r^2 graphical representations have been obtained which might lead one to believe the protein is predominantly dimeric in solution. It is further unlikely that the large fraction of monomer indicated by these results is due to partial degradation of the samples used since $NaDodSO_4$ gel electrophoresis of approximately 20-μg loads of these samples both prior to and following centrifugation showed the presence of only one band of molecular weight 13 500. On the other hand, $NaDodSO_4$ gel electrophoresis of a sample of L7/L12 isolated by the ethanol–ammonium chloride extraction procedure clearly showed the presence of three smaller molecular weight components in addition to the L7/L12 band at similar initial loads.

Although several other studies have reported the results of sedimentation equilibrium experiments with L7/L12 (Möller et al., 1972; Wong & Paradies, 1974; Österberg et al., 1976; Gudkov et al., 1977), in only one case have the experiments conducted been described in sufficient detail to be compared with the present study (Möller et al., 1972). Close examination of the data presented by these authors indicates that the results reported presently are not so incompatible with prior observations as might superficially be supposed. The molecular weights of L7/L12 reported here by $NaDodSO_4$–polyacrylamide gel electrophoresis and by sedimentation equilibrium in 6 M Gdn·HCl are identical with those of Möller et al. (1972). In dilute phosphate buffers, at initial protein concentrations of 0.6–0.8 mg/mL, these authors reported molecular weights in the range 19 000–21 000 obtained from ln C vs. r^2 treatments of the data. Furthermore, they reported observing species exceeding molecular weight 40 000 in these preparations, although $NaDodSO_4$ gels indicated apparent homogeneity. Since the molecular weight obtained by such a treatment was approximately twice that of the guanidine hydrochloride molecular weight, they concluded that L7/L12 was probably present in the form of dimers under these conditions. The results presented here, when plotted in the ln C vs. r^2 format, also yield apparently linear graphs giving molecular weight estimates of 18 600–21 000 from the slope. However, the same data treated by methods capable of much greater resolution demonstrate conclusively that the presence of only dimeric species is a very poor representation of the solution behavior of L7/L12. The deviations of the concentration distribution predicted by the dimer model are always 20–40 μm from the actual distribution obtained at every point in the distribution. That self-associating or otherwise inhomogeneous systems can give rise to apparently linear ln C vs. r^2 plots, resulting in deceptive molecular weight estimates, has been discussed (Aune, 1978). It is evident that L7/L12 behavior is a classic example of this phenomenon.

The solution behavior of L7/L12 appears to be that of an equilibrium self-association. This conclusion is supported by two lines of evidence. First, sedimentation equilibrium experiments conducted at different angular velocities yield the same monomer–dimer, dimer–tetramer equilibrium constants within experimental error. The range of variation of the values obtained for the same refolding of L7/L12 at different speeds is the same as that obtained for different refoldings of L7/L12 conducted at the same speed. Second, the results of the gel chromatography of L7/L12 argue very strongly for an equilibrium self-association. Although two peaks are always eluted from Sephadex G-100, several species are always observed in the material comprising these peaks. Moreover, the eluted peaks always appear to be closely coupled in the effluent, further indicating a dynamic interaction among the various species involved.

The equilibrium constants calculated for the material subjected to gel chromatography suggest there are populations of L7/L12 having two different degrees of self-interaction capability. One population represents 60% of the total refolded protein interacting with association constants $K = K_{12} = K_{24} = 3.5 \times 10^4$ M^{-1}, while the other consists of 40% of the refolded material with association constants $K = 9 \times 10^2$ M^{-1}. The combination of these two fractions of material is consistent with the observation of association constants of 2×10^4 M^{-1} in the refolded L7/L12 subjected to sedimentation equilibrium direct from refolding with no prior gel chromatographic separation. Whether this is an artifact of protein isolation and purification under the denaturing conditions employed is not presently known. Experiments are currently under way to determine whether L7/L12 isolated by the ethanol–ammonium chloride extraction procedure (Hamel et al., 1972; Pettersson, 1980) shows the same tendencies.[2] Prior attempts to determine these parameters with nondenatured L7/L12 were hampered by extensive protein degradation occurring during storage in the frozen state.

Although the gel chromatographic results are not accurate enough to uniquely fix the partition coefficients of the dimeric and tetrameric species, some useful upper limits may be derived for these quantities, allowing one to estimate the lower limits for the Stokes' radii of these species. These results suggest some interesting features of the quaternary structure of the various L7/L12 species. First, the Stokes' radius of the L7/L12 monomer, obtained by extrapolation of the gel chromatography data to infinite dilution, is estimated to be 21–23 Å. Since the radius of the spherical molecule of molecular weight 12 200 with a partial specific volume of 0.755 is 15.5 Å, monomeric L7/L12 appears to have a frictional ratio, f/f_0, of 1.6 by gel chromatography. This is quite consistent with the highly elongated nature of the molecule previously reported (Wong & Paradies, 1974; Österberg et al., 1977; Gudkov et al., 1977). The gel chromatography results are also consistent with Stokes' radii of the dimer and tetramer of 26–28 and 28–31 Å, respectively. These results suggest corresponding frictional ratios of 1.6 for the L7/L12 dimer and 1.3 for the L7/L12 tetramer. This suggests that the L7/L12 tetramer is somewhat more compact than either of the other two species.

X-ray crystallographic studies on the carboxy-terminal (residues 53–120) and amino-terminal (residues 1-36) fragments of L7/L12 (Leijonmarck et al., 1980) show this molecule to have an ellipsoidal head region (20 × 20 × 35 Å) and an extended, highly α-helical tail region 75 Å in length. The α-helical tail is suggested to have several regions of unordered structure which could make it a fairly flexible structure (Leijonmarck et al., 1980). Furthermore, the distribution of basic and acidic residues is fairly well polarized such that the amino-terminal region contains most of the acidic residues and the ellipsoidal carboxy-terminal head contains most of the basic residues (Leijonmarck et al., 1980). Furthermore, the distribution of residues on the carboxy-terminal head seems to form a large positively charged surface opposite a fairly large surface of uncharged residues (Leijonmarck et al., 1980).

Qualitatively, the X-ray dimensions of L7/L12 may provide an explanation for the gel chromatography results. The monomer consists of an ellipsoidal head of axial dimensions 35 × 20 Å and a flexible cylindrical tail 10 Å in diameter × 75 Å in length. If the tail region were capable of folding over on itself, it would give the molecule the appearance of an ellipsoid 20 × 75 Å overall to yield an apparent Stokes' radius of 22 Å. Now if two monomers associated in a parallel fashion, but with the center of one head group shifted with respect to the other and approximately 30–40 Å of the tail regions of the two molecules overlapped, a highly elongated species with axial ratio sufficient to give a Stokes' radius of 26 Å could result. Combination of two dimers antiparallel at the head groups with the dimer could then result in a structure of sufficient symmetry and dimension to yield a Stokes' radius exceeding 30 Å while reducing the apparent asymmetry (hence the frictional ratio) relative to the dimer.

[2] It is possible that this fraction of weakly competent L7/L12 could represent chemically modified species (a partially oxidized, a desamido, or an isocyanate form) having a lesser tendency to self-interact due to the chemical modification.

Finally, although oxidation of methionyl residues certainly causes L7/L12 to exhibit strictly monomeric behavior in TMK buffer, it also appears to increase the Stokes' radius of the monomer by about 1–2 Å. The same is apparently true of the monomeric material derived from DMSI cross-linked preparations. The reasons for this apparent "expansion" of L7/L12 are not understood at present. In addition, it is apparent that cross-linking with dimethylsuberimidate does not necessarily trap the various L7/L12 species in static states of association. Although three distinct peaks of cross-linked material are produced, each is still a mixture of species, varying from heavily enriched in monomer with no tetramer at one extreme to heavily enriched in tetramer with no monomer present at the other extreme. The differences in effective molecular radius of each of these species indicated by these studies are great enough that complete separation of the three species should be obtained if the cross-linking completely prevented dynamic interactions. Consequently, although some "trapping" of equilibrium species may be obtained, dynamic interactions between different cross-linked species may still exist.

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