HYDRODYNAMIC STUDIES ON THE *ESCHERICHIA COLI* RIBOSOMAL PROTEINS S8 AND L6, PREPARED BY TWO DIFFERENT METHODS

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1. Introduction

Several studies have been made to examine the shape of individual ribosomal proteins both in situ on the surface of the ribosome and in an isolated state. The immunological approach to establish the number of specific antibody binding sites for individual proteins on the surface of the ribosome has revealed preliminary evidence for the shape of many protein molecules [1,2]. Hydrodynamic studies have previously been performed on proteins S3, S4, S5, S7 and S20 [3,4] and proteins L7/12 [5]. Further information on the shape of these proteins has come from low-angle X-ray diffraction studies with proteins S4 [4,6], L18 and L25 [7] and L7/12 and L10 [8,9] and from neutron scattering experiments with proteins S2, S5 and S8 [10].

In this study proteins S8 and L6 have been investigated by molecular weight determination, sedimentation and diffusion coefficients and viscosity measurements. Both proteins have been prepared by two different methods: one involving the use of urea at low pH and the other using non-denaturing conditions avoiding the use of urea. Protein S8 prepared by both methods was found to be a compact spherical molecule whereas protein L6 appeared to have a slightly elongated shape. A difference in sedimentation was observed between the two L6 preparations showing that the protein isolated under non-denaturing conditions was more compact. Both proteins showed a reversed solubility effect when prepared by the two different methods.

2. Materials and methods

Proteins were prepared according to the following two methods:

(1) In the presence of urea and acetic acid [11,12].
(2) In the absence of urea, acetic acid and other potentially denaturing conditions at all steps of the isolation [13,14].

The proteins were extracted from the ribosomal subunits into several groups by a stepwise increase of LiCl at neutral pH. Further purification was achieved by chromatography on CM-Sephadex C-25 and Sephadex G-100 in the presence of salt and in the pH-range 5.5–8.0. Purified proteins were concentrated by pressure ultrafiltration or dialysis against dry Sephadex G-150. The identity and purity of the proteins was established by two-dimensional gel electrophoresis [15] and by dodecylsulphate slab-gel electrophoresis [16,17].

Protein concentrations were determined by three methods:

(1) A fluorescamine assay using lysozyme as a standard [18].
(2) Hydrolysis of aliquots of the protein solution with 6 N hydrochloric acid containing 0.2% 2-mercaptoethanol under nitrogen at 110°C for 20 h, followed by amino acid analysis on a Durrum D-500 analyzer.
(3) A nitrogen assay [19] using ammonium sulfate as a standard and calculation of the nitrogen content of each protein from its amino acid sequence [20,21].

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Good agreement was found between methods (2) and (3) for protein determination, hence the values from these methods were used in all calculations. The fluorescamine assay gave higher values for the protein concentration for both proteins S8 and L6.

Experiments with the two S8 proteins were carried out in a buffer containing 0.6 M lithium chloride, 0.05 M sodium acetate, pH 5.6, 0.2 mM dithioerythritol (DTE) and the protease inhibitors benzamidine (BAM, 20 μM) and phenylmethyl sulphonyl fluoride (PMSF, 10 μM). The buffer used for measurements of L6 contained 0.35 M potassium chloride, 0.02 M magnesium chloride, 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), pH 7.0, 1.0 mM DTE, 100 μM BAM and 20 μM PMSF. The protein solutions and their dialysates were filtered through Millipore filters (0.45 μm pore size) before measurements were made. The proteins were checked for degradation after each experiment using the sodium dodecylsulphate slab-gel method [16,17].

Sedimentation equilibrium measurements were made at 20°C ± 0.1°C using a Beckman Model E analytical ultracentrifuge equipped with a photoelectric multiplex scanner and Rayleigh interference optics. Molecular weights were determined by the method of Yphantis [22]; the weight average molecular weight (M_w) was calculated using a least squares fit analysis of ln c versus r^2 and the experimentally determined partial specific volume, $\bar{\nu}$.

Sedimentation velocity experiments were performed at a speed of 56 000 rev./min and at several protein concentrations. For accurate determination of the weight average sedimentation coefficient the second moment position ($\Lambda\bar{x}$) was determined according to Schachman [24].

The diffusion coefficient of the proteins was measured using a capillary type synthetic boundary centerpiece in the AnH rotor of the Beckman Model E ultracentrifuge.

Sedimentation coefficients were extrapolated to zero protein concentration to obtain the $s^0_{20,w}$; diffusion coefficients were averaged to obtain $D^0_{20,w}$ since the experimental error was too large to permit extrapolation.

Viscosity measurements were made with an Ostwald type microcapillary viscometer coupled to an automatic viscosity measuring system (AVS/G, Schott Glass Co., Mainz, FRG), with a flow rate for water of 100 s and at a temperature of 20°C ± 0.01°C. Dilutions of the protein solution were made in the viscometer and an aliquot was removed before each dilution to be used for density and concentration determination. The densities of the protein solutions and their dialysates were measured using a precision digital density meter (DMA 02C, Anton Paar) at a temperature of 20°C ± 0.05°C. Density measurements were analysed by a linear least square fit method. The partial specific volume, $\bar{\nu}$, was determined from the density data using the apparent specific volume [25].

Molecular weights were also calculated from the Svedberg equation using the sedimentation coefficient, partial specific volume and diffusion coefficient.

3. Results and discussion

Sedimentation equilibrium experiments gave molecular weight values that agreed with those obtained from the amino acid sequence [20,21], namely 12 257 for S8 and 18 839 for L6.

Figure 1 shows sedimentation equilibrium experiments with the four proteins. These plots, obtained at low protein concentrations, indicated that the proteins were mainly monomeric. However, varying amounts of dimer and higher aggregates were observed with proteins prepared in urea and acetic acid (S8–urea and L6–urea) at concentrations exceeding 1 mg/ml.

Sedimentation studies with proteins S8 did not reveal a difference in the value of $s^0_{20,w}$ for proteins prepared by either of the two methods, whereas an appreciable difference was observed for proteins L6 (table 1). A smaller $s^0_{20,w}$ value was obtained for L6–urea than for the L6 protein prepared by the non-denaturing method (L6–LiCl). Protein L6–urea also showed an additional fast sedimenting (6 S) boundary due to protein aggregation.

The diffusion coefficients for proteins S8 and L6 are shown in table 1. In both cases a lower value was obtained for the proteins prepared with urea and acetic acid. This was more pronounced in the case of L6.

Molecular weights calculated using the Svedberg equation agreed with the values obtained from sedimentation equilibrium (table 1).

The intrinsic viscosity values for proteins S8

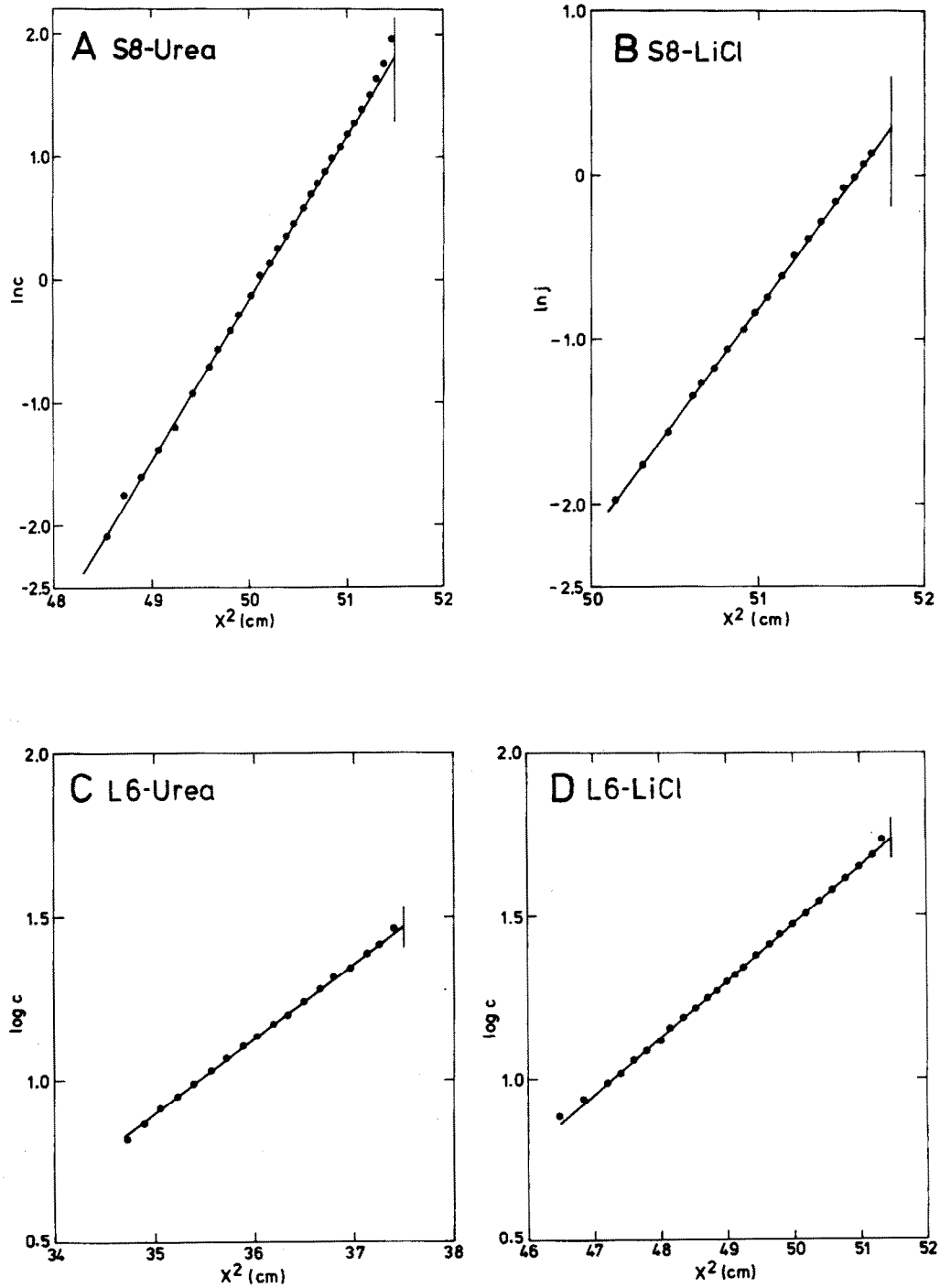

Fig.1. Sedimentation equilibrium patterns of ribosomal proteins S8 and L6. Logarithm of concentration versus the square of radial position. Interference optics (B) and ultraviolet scanner at 280 nm wavelength (A,C,D) were used. The protein samples were centrifuged at 40 000 rev./min (A and B) and 20 000 rev./min (C and D) at a concentration of 0.75 mg/ml.

Table 1
Physical properties of ribosomal proteins

Proteins	$s^0_{20,w}$	$D^0_{20,w} \times 10^{-7}$ (cm^2/s)	$\bar{v}$ (ml/g)	η(ml/g)	Molecular weight		R_S(Å)	f/f_o	$f/f_{o,asym}$	Shape factor	Axial ratio (OE)
					SE	S and D					
S8–Urea	1.61	9.2	0.730	2.5–3.5	13 000	16 000	17.8	1.16	1.02	2.3–3.2	2.3 : 1
S8–LiCl	1.62	9.7	0.730	2.5–3.5	13 400	15 500	18.5	1.17	1.06	2.3–3.2	2.3 : 1
L6–Urea	1.46	7.2	0.735	5.4–5.8	18 500	19 500	26.5	1.52	1.34	4.9–5.3	5.0 : 1
L6–LiCl	1.78	9.2	0.740	4.6–5.0	19 000	19 000	22.1	1.25	1.11	4.2–4.6	4.2 : 1

SE= from sedimentation equilibrium; S and D = from Svedberg equation; OE = oblate ellipsoid. Shape factors and axial ratios were obtained from intrinsic viscosity data. Stokes radius (R_S) and f/f_o were determined according to Tanford et al. [23].

prepared by the two methods were similar (table 1) although S8–urea showed a pronounced concentration dependency above 1 mg/ml which was not seen for the S8–LiCl protein. A slightly lower intrinsic viscosity value was obtained for L6–LiCl than for L6–urea (table 1), the latter sample also showed some concentration dependency.

The amount of hydration of the proteins was estimated from the amino acid composition [26]; a minimum value of 0.35 g H_2O/g protein was used to calculate the shape factors (Simha factor) [27]. Proteins S8–urea and S8–LiCl gave shape factors of 2.8 and proteins L6–urea and L6–LiCl of 5.1. and 4.4 (table 1). Assuming an oblate ellipsoidal shape for both pairs of proteins, these factors lead to axial ratios of 2.3 : 1 for S8–urea and S8–LiCl, and of 5.0 : 1 for L6–urea and 4.2 : 1 for L6–LiCl. The frictional coefficient ratio (f/f_o) and the asymmetry factor ($f/f_{o,asym}$) are shown in table 1. These values show protein S8 from both preparations to have a more spherical structure than protein L6. This compact structure of protein S8 is in agreement with earlier results, obtained from antibody binding studies [1,2] and neutron scattering experiments [10] in situ. Typical values for f/f_o for globular proteins are in the range of 1.05–1.3 [28] hence protein L6–urea appears to have a more extended hydrodynamic structure which could represent an irreversible transition of the native structure which has occurred during the preparation and which is not restored after redissolving the protein in non-denaturing conditions.

The sodium dodecylsulphate gels showed no degradation of proteins S8–urea and S8–LiCl during the course of the experiments, although the former protein always showed two closely migrating bands on the gel, the lower of which co-migrated with protein S8–LiCl. The extra component in protein S8–urea could be due to chemical modification occurring during the purification procedure and is under investigation. Proteins L6 showed slight proteolytic degradation both before and after measurements were made. Since this degraded material represented only 5% of the total protein this was ignored for the purpose of this study.

In a recent study on histone denaturation [29] it was found that the *s*-values for urea treated histone and salt extracted histone were 1.46 S and 1.67 S, respectively. These values give some appreciation of the differences that might be observed for the s values of native and denatured ribosomal proteins, although this will probably also depend on the original shape of the proteins. The difference in s values between proteins L6–urea and L6–LiCl is in the same order of magnitude. Since S8 was found to be a spherical molecule it is possible that the effects of denaturation are more subtle and not so readily detected by hydrodynamic methods.

Ribosomal proteins prepared with urea and by salt extractions showed a different solubility behaviour. Proteins prepared by the former method were very soluble at low ionic strength and much less soluble at high ionic strength while proteins prepared by the non-denaturing method were much more soluble at high salt concentrations. Proteins prepared by both methods had a tendency to aggregate although this was more pronounced with the urea treated proteins. The low solubility at high salt concentrations is probably due to a largely irreversible conformational change during exposure to urea and acetic acid resulting in exposure of previously buried hydrophobic residues. Upon removal of the denaturing agents the original conformation is not restored and hydrophobic groups remain exposed causing aggregation and reduced solubility at high ionic strength. The use of ribosomal proteins in the reconstitution of biologically active small and large subunits [30,31] does not contradict the previous statements. Under reconstitution conditions the proteins are incubated at low concentrations and elevated temperatures in the presence of other ribosomal components and are able to undergo the necessary conformational changes to the 'native' structures. In contrast, the urea treated proteins used in this study were dissolved at relatively high protein concentrations, which are necessary for physical studies, and are probably unable to renature because of aggregation. They were purposely not subjected to renaturation procedures since we wanted to study the effect of the isolation procedure on protein conformation. Also, it was found that acetic acid extracted ribosomal proteins were more active in reconstitution experiments when subjected to a renaturation step in 6 M urea [31]. In a similar manner Rohde et al. [3], to obtain ribosomal proteins in a 'native' state, unfolded the structure with guanidinium hydrochloride before incubation at 37°C in reconstitution buffer.

Solubility effects as observed for ribosomal proteins have also been described for histones [32]. For histone purification it has been necessary to find new purification procedures [33] where the proteins were not subjected to the usual denaturing conditions. The tendency of previously prepared histones to form large unspecific aggregates is believed to be due to the denaturing treatment. Purification of histones under mild conditions has enabled their arrangement in chromatin to be elucidated [34].

We have recently reported [35,36] that when ribosomal proteins were prepared under non-denaturing conditions new RNA binding proteins were found, and the previously established binding proteins bound strongly and without variability to the ribosomal RNA. In contrast, different preparations of protein S8–urea have shown a variation in their ability to bind to 16 S RNA (R. A. Garrett, personal communication). Also, from NMR- [37] and CD-studies [38] it has become clear recently that proteins S8 and L6 prepared with LiCl have retained more of their native structure than the corresponding proteins prepared with urea.

We propose that proteins isolated from the ribosome under non-denaturing conditions have a more compact structure which represents a more native conformation than those proteins extracted in the presence of urea.

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