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UNFOLDED 30 S RIBOSOMAL SUBUNITS

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The 30 S ribosomal subunit from *Escherichia coli* was unfolded into discrete particles upon which physical studies were carried out. These particles were found to be homogeneous and were characterized using sedimentation velocity, diffusion, density and viscosity measurements. The results of these studies clearly certify two distinct stages of unfolding, neither involving a significant loss of protein. However, the results also clearly show that the measurement of only one characteristic (e.g., the sedimentation coefficient) is not sufficient to suggest a structural change. The significance and importance of the apparent specific volume are stressed.

1. Introduction

The present lack of information about the internal organization of the ribosome is one of the major stumbling blocks to a more detailed knowledge of the process of protein synthesis. One possible method to gain a clearer picture of the molecular interactions within the ribosome involves the unfolding of the ribosomal subunits by removal of the Mg^{2+}. This can be accomplished in a number of ways, but especially by using either low Mg^{2+} concentrations [1–4] or by chelation with EDTA [5–10]. The unfolding apparently involves no loss of protein or RNA and is at least partially reversible [2,5,6].

The unfolding process is generally characterized by an observed decrease in the sedimentation coefficient. In the case of the 50 S subunit of the *Escherichia coli* ribosome, which has been the most extensively studied, the unfolding apparently occurs in a series of distinct steps having approximate sedimentation coefficients of 40 S, 30 S and 20 S [2,5,6,11]. The unfolding of the 30 S subunit is not as well defined. Gesteland [5] measured an $s^0_{20,w}$ value of 16 S for the unfolded particle he characterized in a low ionic strength buffer system containing EDTA. Others have reported $s^0_{20,w}$ values of 17–20 S for some unfolded particles, with sedimentation coefficients as low as 7 S being reported in some cases [6,9]. Unfolded subunits have also been characterized by their viscosity [6,8] and thermal stability [7,12]. The effect of unfolding on the physical structure of the ribosomal subunits has also been studied by electron microscopy [1], ORD and CD [9], and small-angle X-ray scattering [3,10].

The purpose of this study was to characterize a single unfolded state of the 30 S ribosomal subunit of *E. coli* by determining a number of its physical characteristics. These values can then be compared with similar measurements of the 30 S subunits in an effort to define more clearly the structure of the subunit and the physical meaning of the process of unfolding.

In the process of this research, it was found that some unexpected changes took place when the ionic conditions were varied only slightly. A discussion of the implication of such changes is also made.

2. Materials and methods

2.1. Preparation of ribosomes

Ribosomes were prepared from mid-log phase *E. coli* MRE 600, purchased from Grain Processing Company, Muscatine, IO, essentially using the

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method of Hill et al. [13]. The separation of 30 S and 50 S subunits was carried out on a 10–30% sucrose gradient containing 100 mM KCl, 10 mM Tris-HCl, pH 7.4, and 1.5 mM $MgCl_2$ in a Ti-15 Beckman zonal rotor with B-29 core at 31000 rpm for 14 h. The 30 S subunits were recovered from the sucrose by precipitating with 2 volumes of cold 95% ethanol. The solution was made 1 mM dithiothreitol and 10 mM $MgCl_2$ prior to ethanol precipitation. The precipitate was collected by centrifugation at 12000 g for 10 min, dissolved in 30 S buffer (1.5 mM $MgCl_2$, 70 mM KCl, 10 mM Tris-HCl, pH 7.4) and dialyzed against the same buffer overnight. Each solution was analyzed by sedimentation velocity experiments for homogeneity. The 30 S subunits were used immediately or stored at −70°C for future use.

2.2. Preparation of unfolded particles

Four different buffers were used to characterize the unfolded particles. Solutions of 30 S ribosomes were dialyzed at 4°C against 4 changes of buffer for a minimum of 36 h. The buffers used were as follows: buffer A (0.1 mM MgCl, 100 mM KCl, 10 mM Tris-HCl, pH 7.4), buffer B (0.1 mM $MgCl_2$, 70 mM KCl, 10 mM Tris-HCl, pH 7.4), buffer C (10 mM EDTA, 70 mM KCl, 10 mM Tris-HCl, pH 7.4) and buffer D (10 mM EDTA, 10 mM Tris-HCl, pH 7.4). The solutions were judged to be homogeneous by sedimentation velocity experiments.

2.3. Sedimentation velocity

The sedimentation velocity experiments were carried out on a Beckman Model E ultracentrifuge utilizing schlieren optics. The studies were made using 12 and 30 mm centerpieces in the appropriate rotors at various speeds between 48000 and 60000 rpm at 4°C. The data were analyzed conventionally by following the maximum ordinate of the boundary using a Nikon 6 C microcomparator equipped with IKL digital micrometers. Data were corrected to water at 20°C and concentrations were corrected for radial dilution. The sedimentation coefficient ($s^0_{20,w}$) was determined by a linear extrapolation to infinite dilution of 4–6 $s_{20,w}$ values at different concentrations.

2.4. Density increment

To determine the density increment ($\partial\rho/\partial c$) of the unfolded particles, the densities of the dialysate and of the ribosomal solutions were determined at 4 ± 0.005°C using a Paar DMA-02C digital density meter interfaced to a Wang 600 calculator. The calibration constant of the instrument was determined with 3 different KCl solutions of density previously determined with 25 ml pycnometers at the same temperature. Stock solutions of 5–15 mg/ml of 30 S ribosomes were filtered through a 0.45 μm Millipore filter and dialyzed against the proper buffer. Following dialysis, the concentration of the stock solution was determined spectrophotometrically using duplicate samples. A weighed dilution series was then made on the stock sample with the dialysate as the diluent, and the density of each sample in the dilution series was determined. The concentration of the dilution series was calculated on a volume dilution basis. On some samples, a dilution series was made previous to dialysis. The concentration of each sample was then determined from A_{260} or by dry weight analysis [14]. The slope of the linear plot of ρ versus c then provided $\partial\rho/\partial c$. The apparent specific volume, Φ^*, was calculated from the following equation: $\Phi^* = [1 - (\partial\rho/\partial c)]/\rho^0$ where ρ^0 is the density of the dialysate.

2.5. Extinction coefficient

The absorbance at 260 nm was determined on duplicate samples with a Cary 15 spectrophotometer (using dialysate as a blank). Dry weight measurements of the sample and dialysate [14] were always made in duplicate or triplicate.

2.6. Diffusion coefficient

The diffusion coefficient was determined from laser light-scattering measurements using the technique of intensity fluctuation spectroscopy. The correlation function was obtained using a Malvern 4300 spectrometer system incorporating a 24 channel digital autocorrelator with a Spectra-Physics 124A He-Ne laser for the incident light. Our system utilized an 8080 microcomputer as an inter-

face to the University computer and a graphics plotter for immediate data analysis. The scattering sample was freed of dust by centrifugation followed by filtration through a 0.45 μm Millipore filter into a 10 mm square fluorometer cuvette. The cuvettes were precleaned with acid, rinsed with double-distilled water and dried with filtered nitrogen. Sample concentrations were varied between 0.5 and 5 mg/ml, but no concentration dependence of D was noted.

Experimental parameters for the photocount correlation experiment were optimized according to Hughes et al. [16]. The sample time was chosen so that the measured correlation function had a span of about two optical coherence times, with an average photocount rate between 1 and 3, and the average clipped photocount rate was ≈ 0.5, for a total number of samples of 10^6-10^7. Routinely, 10 or more measurements were made and the data were independently normalized and then averaged for final determination of D. All experiments were made at 4 ± 0.05°C and at a scattering angle of 90°. The alignment of the spectrometer was checked with Ludox, a gift from the laboratory of H. Eisenberg (Rehovot). The diffusion coefficients were temperature corrected to 20°C in the usual manner.

The first-order electric correlation field for monodisperse spherical particles is

$$g^{(1)}(\tau) = \exp(-\Gamma\tau),$$

where τ is the delay time, and $\Gamma = DK^2$, where K is the scattering vector and D is the translational diffusion coefficient.

The normalized second-order correlation function, $g^{(2)}(\tau)$, as calculated from the single-clipped correlation data, is related to $g^{(1)}(\tau)$ by

$$g^{(2)}{}_\tau = 1 + A\left[g^{(1)}(\tau)\right]^2.$$

A weighted least-squares analysis of $\ln[g^{(2)}(\tau) - 1]$ versus τ yields D where A is a constant which incorporates the effects of spatial coherence [17]. For a polydisperse system of macromolecules, $g^{(1)}(\tau)$ can be written as a sum or distribution of exponentials:

$$g^{(1)}(\tau) = \int_0^\infty G(\Gamma)\exp(-\Gamma\tau)\,d\Gamma,$$

where $G(\Gamma)$ is the normalized distribution of decay rates. An expansion of the logarithmetic expression becomes

$$\ln\left[g^{(2)}(\tau) - 1\right] = \ln A - 2\bar{\Gamma}\tau + \frac{\mu_2}{\bar{\Gamma}^2}(\bar{\Gamma}\tau)^2 - \frac{2}{3!}\cdot\frac{\mu_3}{-3}(\bar{\Gamma}\tau)^3 + \ldots,$$

where μ is a moment about the mean of $G(\Gamma)$. $\bar{\Gamma}$ was found by a weighted polynomial fit of the data [18] from which the Z-average diffusion coefficient was calculated. We have also employed a first-derivative plot of the above relationship as described by Pusey et al. [17]. This plot gives a visual display of fit to a single exponential. We used their quality parameter Q with a value of ≤ 0.02 to define that single exponential.

2.7. Acrylamide gel electrophoresis

The preparation of proteins for the acrylamide gel electrophoresis was done as described by Giri et al. [11]. The two-dimensional gel electrophoresis was performed using the method of Howard and Traut [19] as modified by Giri et al. [11].

2.8. Protein and RNA determinations

The protein concentration on the unfolded particles was determined by the Lowry method [20] using crystalline lysozyme protein as standard. Ribosomal RNA concentration for the unfolded particles was determined by the orcinol method [21] using purified rRNA as the standard.

2.9. Viscosity

The intrinsic viscosity was determined using a Cannon–Ubbelohde semi-microviscometer with a flow time of 230 S for H_2O at 20 ± 0.002°C. The concentrations of the samples were determined spectrophotometrically. Solution and solvent densities were measured with the density meter.

3. Results

3.1. Sedimentation coefficients

After dialysis of 30 S subunits against the four buffers (A–D), the sedimentation coefficients were

found to be significantly different (fig. 1). The particle derived from dialysis against buffer A has an $s^0_{20,w}$ value of 25.7 S and that from buffer B a value of 23.3 S, this difference being due to a 30 mM decrease in KCl concentration holding the magnesium concentration at 0.1 mM.

Dialysis of 30 S subunits against EDTA-containing buffer C gave a particle with an $s^0_{20,w}$ value of 22.8 S. Dialysis against buffer D, which contains no added salts other than those present with Tris or EDTA gives a further reduced $s^0_{20,w}$ value of 19.8 S. These values are all listed in table 1.

3.2. Diffusion coefficients

The diffusion coefficients for the unfolded particles in the four buffers are given in table 1. These results were very different from those expected from analyzing the sedimentation coefficients of the particles. The sedimentation coefficients led us to believe that we had isolated three particles with significant differences in their conformation; namely the 25.7 S and (22.8–23.3)S and the 19.8 S particle. The diffusion coefficients did not substantiate this hypothesis. For instance, the diffusion coefficients for the 25.7 S and 23.3 S particles were essentially the same, indicating the particles had similar frictional coefficients. The diffusion coefficients for the EDTA-derived 22.8 S and 19.8 S particles were significantly lower, indicating further loosening of the particles when placed in these buffers. The unfolded particles were found

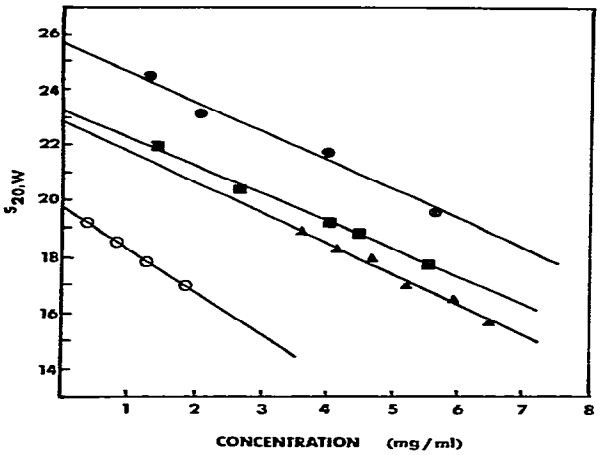

Fig. 1. An extrapolation to infinite dilution of the sedimentation coefficients found for unfolded particles: buffer A (●), buffer B (■), buffer C (▲), and buffer D (○).

to be homogeneous and monodisperse as shown by the linear relationship between $\ln[g^{(2)}(\tau) - 1]$ and τ for the 23.3 S particle in fig. 2.

3.3. Density increments

The density increment, $\partial\rho/\partial c = 0.411$, and the derived apparent specific volume, $\Phi^* = 0.586$ ml/g, for the 25.7 S particle agree with the values of $\partial\rho/\partial c = 0.407$ and $\Phi^* = 0.591$ ml/g determined for the 30 S subunit [14]. The 23.3 S particle does

Table 1

Physical parameters of unfolded 30 S ribosomes

Buffer [a]	$s^0_{20,w}$	$10^7 D_{20,w}$ (cm^2/s)	$\partial\rho/\partial c$	Φ^* (ml/g)	$[\eta]$ (ml/g)	$10^{-5} M$
1 mM $MgCl_2$ 70 mM KCl	31.6	2.10	0.407	0.591	8.10	9.01
0.1 mM $MgCl_2$ 100 mM KCl	25.7	1.72	0.411	0.586	–	8.86
0.1 mM $MgCl_2$ 70 mm KCl	23.3	1.70	0.379	0.618	11.0	8.77
10 mM EDTA 70 mM KCl	22.8	1.58	0.392	0.605	12.2	8.97
10 mM EDTA	19.8	1.52	0.391	0.608	–	8.12

[a] All buffers contain 10 mM Tris-HCl, pH 7.4.

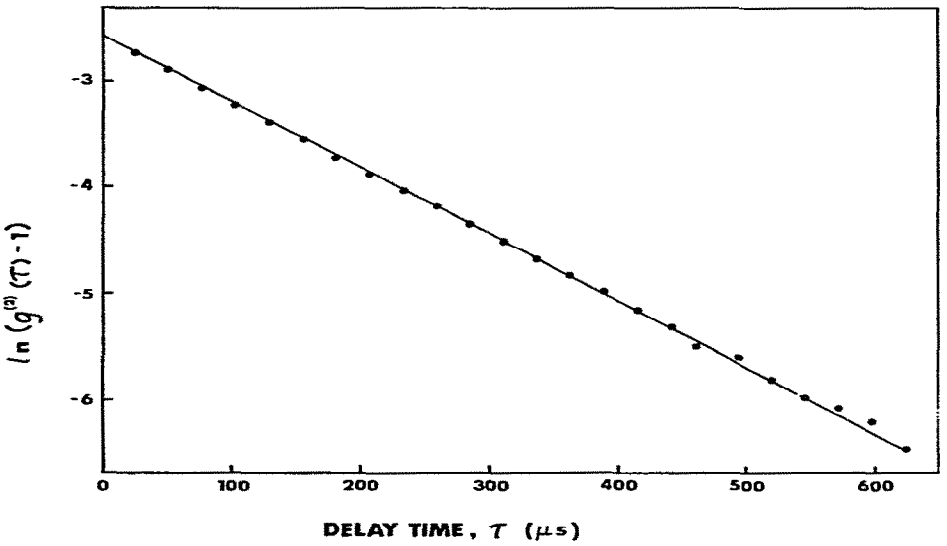

Fig. 2. Correlator output for a sample of 23.3 S particle. The solid line represents the weighted, linear least-squares fit of the data.

show a significant decrease in $\partial\rho/\partial c$, giving a value of 0.379. The buffers for the 25.7 S and 23.3 S particles differ only by 30 mM KCl, but we observe an 8% decrease in $\partial\rho/\partial c$ (0.411 and 0.379) which accounts for the rather large change in sedimentation coefficient. The EDTA-derived particles have similar $\partial\rho/\partial c$ values, but these differ from the other particles. Both $\partial\rho/\partial c$ and Φ^* values for all particles are given in table 1. Fig. 3 shows ρ versus c plots for all particles.

3.4. *Intrinsic viscosity*

The 30 S subunit has an intrinsic viscosity of 8.1 ml/g [14] and a shape approximating an oblate ellipsoid with $\approx 4:1$ axial ratio [13]. Upon unfolding there was a dramatic increase in the intrinsic viscosity of the particles. For the particle in buffer C (22.8 S particle) a value of 12.2 ± 0.5 ml/g was measured while with the particle in buffer B (23.3 S particle) a value of 11.0 ± 0.5 was found (see fig. 4). Viscosities were not measured for the other two particles due to lack of material.

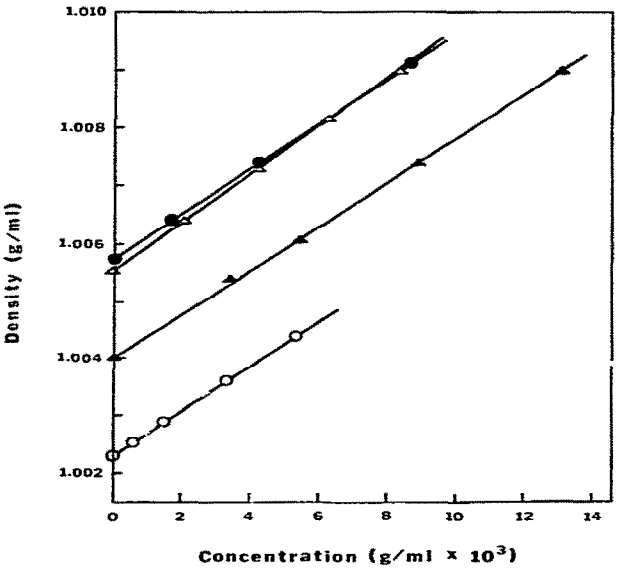

Fig. 3. Densities of 25.7 S (●), 23.3 S (□), 22.8 S (▲) and 19.8 S (○) unfolded particles.

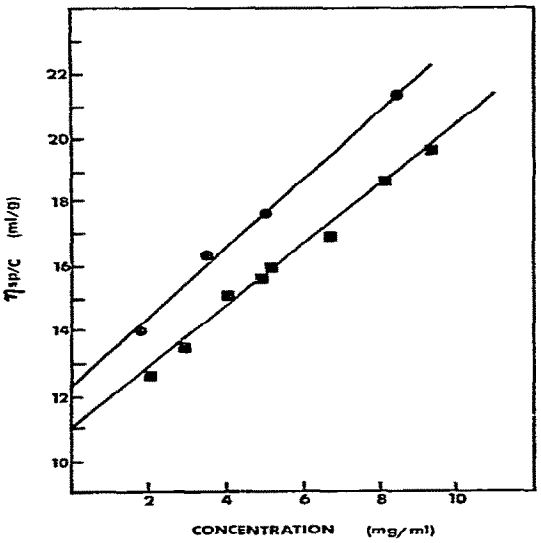

Fig. 4. An extrapolation to infinite dilution of the reduced viscosities found for 23.3 S (■) and 22.8 S (●) unfolded particles.

3.5. Protein and RNA content

Chemical analysis using the orcinol and Lowry methods showed that the 30 S subunits and the 22.8 S and 23.3 S unfolded subunits had a protein-to-RNA ratio of 0.43 ± 0.03. This ratio is equivalent to 31 (± 2)% protein and 70 (± 2)% RNA. In addition, two-dimensional acrylamide gel electrophoresis showed that all of the proteins normally present on washed 30 S ribosomal subunits were present on all four of the unfolded particles.

3.6. Molecular weights

The molecular weights of the unfolded particles were calculated by combining sedimentation and diffusion coefficients with the density increment in the Svedberg equation

$$M = \frac{s^0_{20,w} RT}{D^0_{20,w}(\partial\rho/\partial c)}.$$

We have assigned a 5% error to these values (see table 1), since the estimated error in the diffusion coefficient is 2–3% and that of $\partial\rho/\partial c$ is $\simeq 1\%$. Except for the 19.8 S particle, the values for the molecular weights of the unfolded particle are identical, within error, to the value determined for the 30 S subunit.

3.7. Extinction coefficients

The extinction coefficient ($E^{1\%}_{260}$) for the 23.3 S particle was found to be 145 ± 3, essentially the same value as measured for the 30 S subunit [14]. The extinction coefficient was not measured for the 25.7 S particle but was assumed to be the same as that of the 23.3 S particle. The two EDTA-derived particles have a measured value of $E^{1\%}_{260} = 140 \pm 3$. Other workers have reported no significant change in the extinction coefficient of EDTA-derived particles.

3.8 Derived results

From the values obtained for the diffusion coefficients, sedimentation coefficients, viscosities, density increments and molecular weights, we can calculate various hydrodynamic parameters for each particle.

Since there is no accurate, direct way to measure the water of hydration, we have used a value obtained from small-angle X-ray scattering (1.3 g H_2O/g RNA) by Smith [10]. Using this, the axial asymmetry can be calculated, assuming an appropriate shape. We have assumed a prolate ellipsoidal shape in all cases.

The Sheraga-Mandekern shape factor (β) can also be calculated for those particles for which viscosity values were obtained. The values for the 23 S particles were surprising, since they were less than 2.12×10^6, the putative minimum for any ellipsoidal structure. The physical implications of these results are not clear.

Although there are many other values that can be calculated using the measured parameters, such as R_h, f/f_{min}, etc., the physical meaning of such values is unclear, since the particle shape almost certainly does not conform to a standard geometrical model. Therefore, we have included only the values for f/f_0, a/b (the axial ratio) and β in table 2.

Table 2

Derived physical parameters of unfolded 30 S ribosomes

Particle (s)	f/f_0 ($\delta = 1.3$)	a/b (prolate)	$10^{-6}\beta$
31.6	1.14	3.6	2.14
25.7	1.44	8.0	–
23.3	1.44	8.2	1.93
22.8	1.55	10.3	1.87
19.8	1.66	12.3	–

4. Discussion

The results of this study clearly show the need to measure several physical parameters of macromolecules in order to ascertain conformational changes that may be occurring in various ionic conditions. For instance, in this study the sedimentation coefficient measurements alone suggested that the 25.7 S, the (23.3–22.8)S and the 19.8 S ribosomal particles were three structurally different species. Yet diffusion and density increment results indicated that the difference between the 25.7 S particle and the 23.3 S particle was due to a change in the density increment, whereas there was apparently considerable structural loosening between the 23.3 and 22.8 S stages. Thus, using sedimentation coefficients alone to evaluate structural changes could lead to erroneous conclusions.

There was an additional difference noted between the 22.8 S and 23.3 S particles. Upon dialysis of the 23.3 S particle into buffer A, which contained 100 mM KCl rather than 70 mM KCl, the sedimentation coefficient was raised to 25.7 S. This appeared to be a completely reversible process. However, the sedimentation coefficient of the EDTA-derived 22.8 S particle was not altered upon dialysis against buffer A. These differences in reversibility would tend to substantiate that the 22.8 S and 23.3 S particles are quite different and would suggest structural differences between them.

The ionic conditions for the 22.8 S particle compared favorably with those which Eilam and Elson [9] used to isolate a particle with similar sedimentation characteristics. However, the 19.8 S sedimentation coefficient was considerably higher than the 16 S value Gesteland [5] found in a similar buffer, the only difference being that he used 1 mM EDTA whereas we used 10 mM EDTA. Perhaps the difference is entirely due to the difference in the ionic strength which can greatly aggrevate non-ideality problems at that level. Exact comparisons between the particles we isolated and those isolated by others are quite difficult due to the dearth of physical data from those workers.

The diffusion results obtained in this study are the first reported on unfolded ribosomal particles except for a brief report by Koppel [18] of a diffusion coefficient of 1.78×10^{-7} cm^2/s for a 25 S particle that was present in his 30 S preparations. This value is quite similar to that observed for the 25.7 and 23.3 S particles reported herein.

Comparison of the diffusion coefficients is very useful, since the diffusion coefficient is inversely dependent on the frictional coefficient. However, the frictional coefficient is a measure of both asymmetry and hydration. Nonetheless, as the diffusion coefficients are compared between similar preparations hydration may be assumed to remain essentially constant. Comparison of diffusion coefficients may then allow a direct comparison of the asymmetries of the particles in question, assuming their molecular weights are the same.

Based upon these assumptions, the results of this study indicate that there is substantial loosening initially between the 30 S subunit and the (25.7–23.3)S particles. The additional reduction in the diffusion coefficient between the 22.8 S and 19.8 S particles may merely reflect loss of mass rather than additional structural loosening, since the molecular weight decreases in this transition. All of these changes may be due to the non-ideality problems discussed above.

Perhaps the key to the cause of the variation in the sedimentation coefficients lies with the density increments that were measured. Since

$$s = \frac{M\partial\rho/\partial c}{Nf} = \frac{DM\partial\rho/\partial c}{RT},$$

it is apparent that a change in M, D or $\partial\rho/\partial c$ could alter the sedimentation coefficient. Changes in M do not occur in the particles studied herein, except perhaps for the particle isolated from buffer D. In all other cases, either the diffusion

coefficient changed (suggesting a conformational change) or $\partial\rho/\partial c$ changed to cause the alteration of the sedimentation coefficient.

What does an alteration in $\partial\rho/\partial c$ denote? Since for an ideal two-component system [22]

$$\partial\rho/\partial c = 1 - \bar{v}_2\rho^0,$$

one might be tempted to suggest that the partial specific volume itself was changing. However, since we are dealing with a multi-component system, the observed change in $\partial\rho/\partial c$ is much more difficult to explain. The cause of an alteration of the density increment $\partial\rho/\partial c$ of the ribosomal particle may be due to preferential salt binding, electrostatic shielding, solvent constriction or the specific volume of the ion themselves, all of which can alter the density increment, yet not involve the value of $\bar{v}_2$. Some combination of these many effects is almost certainly the cause of the changes in $\partial\rho/\partial c$ that we observe.

Thus, ionic-environment-induced changes in the density increment can alter the sedimentation coefficient, as is clearly shown in the change from 25.7 S to 23.3 S. The latter particle has a density increment that is 8% lower than that of the 25.7 S particle, a change which was caused by a slight decrease in KCl concentration (30 mM). Since the diffusion coefficient does not change (suggesting no structural change is occurring) the decrease in the sedimentation coefficient is directly attributable to the decrease in $\partial\rho/\partial c$.

Molecular weight changes must also be monitored. This is quite straightforward if S, D and $\partial\rho/\partial c$ can all be measured with sufficient accuracy. In this study, the molecular weights of all of the particles studied, except the 19.8 S particle, were essentially identical. This reemphasizes the point made by previous workers that even when unfolded, ribosomal subunits tend to retain their protein complement although the specific binding of the protein may be altered [23]. Only in the case of the 19.8 S particle was there a molecular weight difference. However, the accuracy of this number must be questioned, since the ionic strength was so low that all of the measurements would suffer from the effects of non-ideality.

The conclusions one can draw from these measurements are both narrow and broad. It is apparent that unfolding of the 30 S subunit does occur under the conditions given above. Careful analysis of table 1 indicates that the unfolding occurs in two rather distinct steps. The initial unfolding takes place when 30 S ribosomal subunits are placed in 0.1 mM Mg^{2+}, the next when the Mg^{2+} is eliminated by chelation with EDTA. However, the change in the sedimentation coefficient does not reflect the magnitude of the second stage of unfolding (23.3 to 22.8 S), since there is a concomitant decrease in the apparent specific volume Φ^*.

On a broader note, the conclusions of this study are much more debilitating. All comparisons of structure must be made under identical ionic and concentration conditions if only one physical parameter is measured (e.g., sedimentation coefficients). Calculations or estimations of the partial specific volumes are to be avoided, especially in the case of polyelectrolytes such as nucleic acids or ribonucleoproteins. Hydrodynamic evidence for structural change cannot be conclusive until all the essential variables are measured under the ionic conditions of the experiment. For instance, apparent specific volumes cannot be assumed constant for macromolecules in different solvent systems. Finally, any conclusions on the structure of the macromolecules themselves must be carefully stated to include those assumptions made to produce the conclusions. For instance, the possible effects of hydration changes, salt effects and ionic strength must be discussed before structural conclusions can be made.

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