Physical Characteristics of Protein-Deficient Particles Derived from the 50S Ribosomal Subunit†

Lallan Giri, Ming F. Tam, and Walter E. Hill‡,*

ABSTRACT: Sedimentation coefficients, diffusion coefficients, density increments, extinction coefficients, and molecular weights were determined for selected core particles from the 50S *Escherichia coli* ribosomal subunits. These core particles were found to be loosened structures containing less than half the protein present on the intact 50S subunits and totally lacking peptidyl transferase and polyphenylalanine synthesis activity unless reconstituted. The results of this study confirm that a loosening of the subunit takes place upon the removal of protein, but this loosening is not necessarily due to the loss of the protein.

In order to study the structure and function of ribosomes, ribosomal subunits have been altered in several ways, including the removal of proteins. Protein-deficient particles were first reported by Meselson et al. (1964) who noted that some protein was removed from ribosomes when they were treated with high concentrations of CsCl. More recent studies from other laboratories have shown that different salts under various ionic conditions will also cause selective removal of different proteins (Itoh et al., 1968; Atsmon et al., 1969; Spitnik-Elson and Atsmon, 1969; Spitnik-Elson and Greenman, 1971; Homann and Nierhaus, 1971). These studies have mainly been directed toward understanding the functional characteristics and determining the specific proteins removed from the various protein-deficient particles, generally termed core particles.

Due to the relative difficulty in isolating homogeneous samples, physical studies have not been made on any of these core particles, except for a small-angle x-ray scattering study on the 23S core particle derived from the 30S subunit (Smith, 1971). The present study was carried out on 31S core particles derived from the 50S subunit. Physical measurements were made in an effort to characterize the conformational change caused by the removal of protein under selected ionic conditions. It is felt that the results of this study, coupled with similar structural studies on other altered subunits, will contribute to the delineation of the subunit structure.

Materials and Methods

Preparation of Ribosomes. Ribosomes were prepared from *Escherichia coli* MRE 600, harvested in the late logarithmic phase, essentially using the method of Hill et al. (1969b). In the final steps, the separation of 50S subunits from the 30S subunits was carried out on 10–36% exponential sucrose gradient prepared in 0.1 M KCl, 0.01 M Tris-HCl, pH 7.4, and 0.0015 M $MgCl_2$ in a Ti-15 Beckman zonal rotor spun at 32 000 rpm (100 000*g*) for 10 h. The 50S subunits were recovered from the sucrose solution by precipitating with 2 volumes of 95% ethanol. The precipitate was collected by centrifugation at 30 000*g* for 30 min, dissolved in a buffer containing 1.5 mM $MgCl_2$, 0.1 M KCl, 0.01 M Tris-HCl, pH 7.4 (core buffer), and dialyzed against the same buffer overnight with two to three changes of dialysate. The purity was checked by means of sedimentation velocity experiments using a Beckman Model E ultracentrifuge. The ribosomal subunits were either used immediately or stored at −70 °C for future use.

Preparation of Core Particles. A solution containing 1 mg/ml of 50S ribosomal subunits in core buffer was incubated with an equal volume of 6 M LiCl, 0.05 M Tris-HCl, pH 7.4, with or without 10 mM EDTA[1] for 24 h at 4 °C, giving a final concentration of the incubation medium of 0.75 mM $MgCl_2$, 3 M LiCl, 0.05 M KCl, 0.03 M Tris-HCl, pH 7.4, with or without 5 mM EDTA. After incubation, the mixture was centrifuged at 50 000 rpm (250 000*g*) in a Ti-60 rotor in Beckman Model L3-50 centrifuge for 4–5 h and the supernatant was saved for split protein preparation. The pellets were resuspended in core buffer and centrifuged at 20 000*g* for 10 min to remove aggregated material. The final purification was performed on a 10–30% sucrose gradient in a Ti-14 zonal rotor at 48 000 rpm (170 000*g*) for 4 h. The core particles were then recovered and concentrated from the fractions by precipitation with 95% cold ethanol and dialyzed against core buffer to remove ethanol. The core particles were further purified by passing through a 1 × 50 cm column of Sephadex G-100. Samples were either used immediately or stored at −70 °C. No sample was frozen and thawed more than once. Partial reconstitution of the core particles was carried out using the method described by Staehelin and Maglott (1971).

Preparation of Proteins. The proteins from 50S intact subunits and protein-deficient particles were extracted with 67% acetic acid in 0.1 M $MgCl_2$ using a modification of the method outlined by Hardy et al. (1969). This mixture was stirred for 45 min at 4 °C. After centrifugation at 30 000*g* for 30 min, the pellet was washed with the same 67% acetic acid mixture and recentrifuged. The proteins in the combined supernatant were precipitated with 10% trichloroacetic acid. The precipitate was collected by centrifugation at 30 000*g* for 30 min, dissolved in 8 M urea, 0.04 M Tris-HCl, pH 4.5, and lyophilized.

The protein concentration on protein-deficient particles was determined by the Lowry test (Lowry et al., 1951) using

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[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; ATP, adenosine triphosphate; PEP, phosphoenol pyruvate.

crystalline lysozyme protein as standard. Ribosomal RNA concentration on protein-deficient particles was determined by the orcinol test (Dische, 1955).

Acrylamide Gel Electrophoresis. The acrylamide gel electrophoresis of core proteins, total protein, and split protein was performed using the method of Howard and Traut (1973), except that 6 M urea was added to the buffer in the first dimension. The compositions of the first- and second-dimension gel electrophoresis as well as the buffer in the second dimension were identical to that described in the literature.

Extinction Coefficient. The extinction coefficient was determined by measuring the absorbance of solutions at 260 nm and then determining the concentration of those solutions by means of dry weight measurements (see Hill et al., 1969a). Two-milliliter solutions previously dialyzed to equilibrium were weighed in 10-ml tared volumetric flasks and dried *in vacuo* to a constant weight at 98–100 °C. The optical densities of duplicate sample solutions at 260 nm were carefully recorded on a Cary 15 spectrophotometer.

Physical Measurements. Sedimentation Coefficients. The apparent sedimentation coefficients of the protein-deficient particles were determined using a Beckman Model E ultracentrifuge at 60 000 rpm and at 4 °C. These values were corrected for temperature and solvent differences and then extrapolated to infinite dilution to give $s^0_{20,w}$ values for the two core particles.

Density Increment. The density increment $(\partial\rho/\partial c)$ (Casassa and Eisenberg, 1964) was obtained from a density vs. concentration plot. The density was determined using a Paar DMA-02C digital density meter. Each sample was filtered through a Millipore filter and dialyzed against core buffer previously filtered through a sintered glass filter. The optical densities of duplicate samples were recorded after each run for concentration determination.

Viscosity. The intrinsic viscosity was determined using a Cannon–Ubbelohde semimicro viscometer with a flow time of 230 s for H_2O at 25 ± 0.002 °C. The concentrations of the samples were determined spectrophotometrically. Solution and solvent densities were measured as described above.

Diffusion Coefficient and Molecular Weight. The diffusion coefficients were determined by intensity fluctuation spectroscopy using the method of Koppel (1974). The sample was banded in a 5–20% sucrose gradient using a Beckman SW 50.1 rotor and run at 50 000 rpm for 2.5 h. The diffusion measurements were made directly on a sample band in the centrifuge tube, using a Malvern digital correlator to measure the intensity fluctuation. The molecular weight was then calculated from the Svedberg equation using the sedimentation coefficient, density increment, and diffusion coefficient.

Assays. Peptidyl transferase activity was measured essentially as described by Monro and Marcker (1967) as modified by Monro et al. (1969). In the present study, the whole fMet-tRNA was used as substrate, instead of the fragment (Monro et al., 1968). In a typical run, a 150-μl reaction mixture contained 50 mM Tris-HCl (pH 7.4), 0.4 M KCl, 20 mM $Mg(OAc)_2$, 1 mM puromycin, 8000–10 000 cpm of [^{3}H]-fMet-tRNA, 2–3 A_{260} units of core particles or 50S subunits, and 25 μl of methanol. The methanol was added last to start the reaction. The mixture was incubated at 0 °C. The [^{3}H]-fMet-tRNA–puromycin complex was extracted in ethyl acetate and the resulting radioactivity was monitored.

Poly(U)-directed phenylalanine incorporation was measured using the method of Gilbert (1963). A 0.2-ml reaction mixture contained 1 mM ATP, 5 mM phosphoenol pyruvate (PEP), 10 μg of PEP kinase, 50 mM KCl, 15 mM $Mg(OAc)_2$, 10 mM Tris-HCl (pH 7.4), 50 μl of S-100 solution, 1 A_{260} unit of 30S and 1.5 A_{260} units of core particles or 50S subunits, 20 μg of poly(U), 0.5 μCi of [^{14}C]phenylalanine, and 3 mM β-mercaptoethanol. The mixture was incubated at 20 °C. The carrier albumin was added in some experiments. The reaction was stopped, precipitated with 5% Cl_3CCOOH and filtered on Millipore filters, and radioactivity was counted in a PPO-Beckman Bio-Solv–toluene scintillation fluid system.

TABLE I: Protein Content of Core Particles.

Sample	EDTA in Incubation Mixture	Protein (%)	RNA (%)	Protein RNA
50S subunits		35	65	0.54
Core particles	0.005 M	13.5	86.5	0.16
Core particles		15	85	0.18

Results

Protein Content of Core Particles. Table I shows the total protein and RNA content of the core particles after 24-h exposure of ribosomal subunits to the incubation medium. When incubation time was increased to 36 or 48 h, more proteins were detached. It was found that the amount of protein being removed is dependent on several factors, such as time, nature and concentration of the monovalent salt, Mg^{2+} concentration, and ribosome history (Spitnik-Elson and Atsmon, 1969).

Two-dimensional gel electrophoresis clearly shows the proteins present on the RNA (core proteins) and those released by the LiCl (split proteins). The results from gels run on three separate samples are recorded in Table II. Addition of EDTA to the incubation medium caused the following differences. (a) There were only 12 proteins on the core particle instead of 13 for the non-EDTA-derived core particle. (b) L19 and L30 were split from the RNA. (c) L29 remained on the core particle.

It was also noted that protein L22 was found in both the core and split protein fractions. Protein L30 was present in the core and split protein fraction of the non-EDTA-derived core, but with EDTA present, was found only in the split protein fraction.

Extinction Coefficient. The extinction coefficients of the core particles were determined as outlined in the preceding section. The results listed in Table III show that the extinction coefficient of the core particles is somewhat greater than that of the 50S subunit, as might be expected due to structural loosening of the core particles. The slight increase of the extinction coefficient of the EDTA-treated core particle is probably due to the additional proteins removed using this treatment, allowing additional exposure of the RNA.

Activity of Core Particles. Polyphenylalanine Synthesis. As shown in Figure 1A, no phenylalanine incorporation was observed in either core particle, indicating that core particles are completely inactive in protein synthesis.

When protein-deficient particles and split proteins were incubated together by using the method described by Staehelin and Maglott (1971), a significant increase in their phenylalanine incorporation ability was observed. However, the resultant activity of these reconstituted particles was not as great as that of the intact 50S subunits. No further studies on the reconstituted particles were made.

Peptidyl Transferase Activity. As described by Monro et al. (1969) using 50S subunits in the presence of alcohol, a reaction between ^{14}C-labeled fMet-tRNA and puromycin to form [^{14}C]fMet-tRNA–puromycin takes place. This [^{14}C]fMet-

TABLE II: Protein Composition of Core Particles.

Protein Number	Incubation Mixture Containing 5 mM EDTA		Incubation Mixture Without EDTA	
	Core Protein	Split Protein	Core Protein	Split Protein
1	+	−	+	−
2	+	−	+	−
3	+	−	+	−
4	+	−	+	−
5	−	+	−	+
6	−	+	−	+
7	−	+	−	+
8	−	+	−	+
9	−	+	−	+
10	−	+	−	+
11	−	+	−	+
12	−	+	−	+
13	+	−	+	−
14	+	−	+	−
15	−	+	−	+
16	−	+	−	+
17	+	−	+	−
18	−	+	−	+
19	−	+	+	−
20	+	−	+	−
21	+	−	+	−
22	+	+	+	+
23	+	−	+	−
24	−	+	−	+
25	−	+	−	+
26	−	+	−	+
27	−	+	−	+
28	−	−	−	+
29	+	−	−	+
30	−	+	+	+
31	−	+	−	+
32	−	+	−	+
33	−	+	−	+

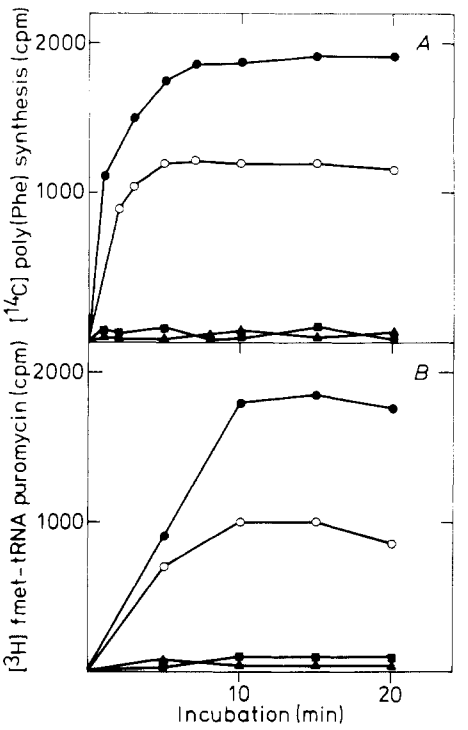

FIGURE 1: Activity studies on the core particles. (A) Poly(U)-directed polyphenylalanine synthesis. (B) Peptidyl transferase activity. Procedures are outlined under Materials and Methods. (●) Intact 50S subunits; (○) core particles and split protein fraction; (▲) core particles with EDTA; (■) core particles without EDTA.

tRNA–puromycin complex, a nonionized component, is extracted into ethyl acetate. Both unreacted fMet-tRNA and the free fMet, an ionized component, are insoluble in this solvent.

As Figure 1B shows, both core particles were unable to catalyze the peptidyl transferase activity or alcohol reaction.

Physical Studies. Sedimentation Coefficient. The sedimentation coefficient, $s_{20,w}$, was extrapolated to zero concentration to give $s^0_{20,w}$ values of 31.2 and 31.9 S for the core particles obtained by incubating with and without EDTA, respectively. The 31.2S particles show slightly more concentration dependency than the 31.9S particles. It was also noted that the 31.9S particles showed a slightly more symmetrical schlieren peak than the 31.2S particle.

Density Increment. The determination of the density increment ($\partial\rho/\partial c$) was essential for an accurate molecular-weight determination. The density increments for the core particles were found to be 0.415 and 0.423 for the core particles prepared with and without EDTA, respectively. These values are only slightly greater than the 0.412 value obtained for 50S subunit. This was somewhat unexpected, since there were rather large structural and ionic-condition differences between the core particles and the native subunits.

Viscosity Studies. Values for $\eta_{sp/c}$ were determined for the two core particles and, upon extrapolating these values to infinite dilution, $[\eta]$ was found to be 15.7 ml/g for the EDTA-incubated particles and 15.1 ml/g for the core particles prepared in the absence of EDTA. These values are considerably greater than the 5.6 ml/g obtained for the 50S subunit, which indicates a considerable conformational and/or volume change. Unlike the reduced viscosity of intact 50S particles which showed virtually no concentration dependence (see Hill et al., 1969a), the reduced viscosity of the core particles showed considerable concentration dependency.

By using these intrinsic viscosities and assuming no change in hydration, the shape factors for the core particles can be approximated from the relation (Van Holde, 1971)

$$[\eta] = \nu(\bar{v} + \delta\bar{v}_0)$$

TABLE III: Comparative Physical Data on Core Particle Derived from 50S Ribosomal Subunits.

Type	$s_{20,w}$	$(\partial\rho/\partial c)$	$E^{1\%}_{260}$	$[\eta]$ (ml/g)	$D_{20,w} \times 10^7$ (cm²/s)	$M \times 10^{-6}$
50S subunits	50.2 ± 0.5[a]	0.412 ± 0.006[a]	145[a]	5.6 ± 0.2[a]	1.9 ± 0.03[b]	1.55 ± 0.05[a]
Core particle	31.9 ± 0.5	0.423 ± 0.005	170	15.1 ± 0.4	1.40 ± 0.04	1.32 ± 0.03
Core particle with EDTA	31.0 ± 0.5	0.415 ± 0.005	180	15.7 ± 0.3	1.37 ± 0.04	1.30 ± 0.03

[a] Hill et al. (1969a). [b] Koppel (1974).

where ν is the shape factor, δ the hydration, and $\bar{v}_0$ the partial specific volume of water. The values for the partial specific volume ($\bar{v}$) can only be approximated using $(\partial\rho/\partial c)$, since we are working with a three-component system in studying the core particles. Using $\bar{v} = 0.577$, $[\eta] = 15.4$ ml/g and assuming $\delta = 1.3$ g of H_2O/g of ribosome, the average value of the shape factor (ν) for the core particles is found to be 8.2, which gives an axial ratio of about 7 assuming an oblate ellipsoidal shape or 10 assuming a prolate ellipsoidal shape. Inasmuch as the shapes of the core particles are almost certainly not ellipsoidal, these ellipsoidal approximations are only useful to indicate the degree of asymmetry occurring upon the removal of the proteins.

Diffusion Coefficient Determination. The diffusion coefficients ($D_{20,w}$) of the two core particles were measured directly on samples banded in a 15–20% sucrose gradient (see Koppel, 1974) to avoid any possible contaminants. The core particles showed a significant decrease in $D_{20,w}$ value as compared to 50S intact particles (Table III). This decrease in $D_{20,w}$ is another parameter indicating a marked conformational change in the protein-deficient particles as compared to the native 50S subunits. The core particles with EDTA had a slightly lower diffusion coefficient than the core particles without EDTA. At the low concentrations used, $D_{20,w}$ was assumed to be equal to $D^\circ_{20,w}$ (see Koppel, 1974).

Molecular Weight. The molecular weights of the core particles were calculated by combining the values for $s^0_{20,w}$, $D^0_{20,w}$, and $\partial\rho/\partial c$ in the Svedberg equation. These results are also posted in Table III.

Discussion

The results of this study definitively show that the removal of proteins using high salt is concomitantly coupled with extensive unfolding of the 50S subunit. Under the incubation conditions used in this study, the 50S subunit is changed from about 2:1 prolate ellipsoid (Hill et al., 1969a; Tolbert, 1971) to a structure having an approximate axial ratio of about 10:1, assuming the hydration remains constant. Although there are many ionic conditions under which core particles can be produced (see Homann and Nierhaus, 1971), the conditions used in this study were selected in order to provide a homogeneous sample upon which physical studies could be made. Nonetheless, it is felt that under other high salt conditions, by which proteins are released, similar loosening of the structure will take place.

Under the incubation conditions used, we found that almost two-thirds of the proteins present on the 50S subunit are released. The number of proteins released depends upon time, ionic strength of the solvent, incubation time, and, probably, the previous history of the subunit as well. Our conditions are similar to those used by Itoh et al. (1968), under which they obtained 28S particles having a similar protein complement. However, we found that addition of 5 mM EDTA did not appreciably reduce the sedimentation coefficient of the core particles, as it did in their study. A rather comprehensive study of the proteins released under various ionic conditions has been reported previously by Homann and Nierhaus (1971). While our core protein profiles do not match theirs exactly, the small differences are almost certainly due to differences in ribosome preparation and/or in the incubation mixtures used.

The molecular weights of the core particles were determined by coupling sedimentation and diffusion measurements, rather than by sedimentation equilibrium experiments. We had persistent difficulty with the latter due to some ubiquitous low-molecular-weight contaminants. If we add the molecular weights of the core proteins to the molecular weight of the 23S rRNA (1.1×10^6), we obtain 1.33×10^6 and 1.35×10^6 for the molecular weights of the EDTA- and non-EDTA-derived core particles, respectively. These values are slightly higher than the respective 1.30×10^6 and 1.32×10^6 dalton values obtained from sedimentation and diffusion measurements, but are still within experimental error.

The change in the extinction coefficient ($E_{260}^{1\%}$) from 145 for the 50S subunit to 170–180 for the core particles is, approximately, that to be expected from the increased exposure of the RNA in the core particles. Considerable care was taken to ensure that EDTA did not interfere with our absorption results.

A surprising result was found upon measuring density increments. Since the unfolding had been so great, it was expected that the density increment would decrease as it has in other unfolded particles (Blair and Hill, unpublished results). However, the results found in this study show only a very slight increase, possibly due to the loss of protein, very high salt conditions, or both. Whatever the reason, a direct comparison of core particle density increments with that of the 50S subunit is not fruitful, since the conditions are so different.

The decrease in the sedimentation coefficient of the core particles from that of the 50S subunit is that expected due to the loss of protein and the unfolding that occurs. The loss of the proteins, assuming no other structural changes, would produce a particle with a sedimentation coefficient of approximately 44 S. The remainder of the decrease in the sedimentation coefficient is due to the unfolding and, perhaps, some hydration changes as well.

The above results should not be interpreted to mean that the loss of protein causes the unfolding of the particle. It is expected that the ionic conditions necessary to release the proteins are much more responsible for the loosening of the subunit structure. For instance, magnesium bridges between adjacent RNA strands are almost certainly broken under the high salt concentrations used. That this can cause extensive unfolding has been shown previously by many workers (Spirin et al., 1963; Gavrilova et al., 1966; Gesteland, 1966; Weller et al., 1968; Wong and Dunn, 1974; Gormly et al., 1971; Eilam and Elson, 1971) in which Mg^{2+} was depleted through dialysis or by chelation with EDTA, resulting in unfolded particles. In these cases, the protein complement remained almost wholly intact.

Atsmon et al. (1969) have previously noted that protein-deficient particles behaved in a manner similar to that of unfolded particles both in their sedimentation behavior and their lack of resistance to ribonuclease. Our results confirm the fact that the unfolding is extensive and is not increased significantly when EDTA is used to chelate the Mg^{2+} ions. Although we have not been able to isolate and physically characterize the equivalent unfolded particle with the full protein complement, there is a discrete 30S peak that appears in the schlieren patterns of unfolded 50S subunits.

It should also be noted that, as in the case of unfolded particles, the various core particles often appear as discrete peaks in a sedimentation velocity pattern. This is indicative of a discrete unfolding, rather than a random loosening of the ribosomal structure. It is expected that further physical studies of both the core particles and the unfolded particles will aid in determining the structural constraints designating this characteristic unfolding pattern.

In conclusion, this study confirms that the protein-deficient core particles are very loose structures as compared with the 50S subunits. This loosening is not directly attributable to the

loss of proteins, but more probably due to the ionic conditions necessary to remove these proteins. This protein-deficient particle is quite stable and is possibly equivalent to the unfolded particle of similar sedimentation coefficient.

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