Conformation of Ribosomal Proteins Free in Solution and Bound to Ribosomal RNA†

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ABSTRACT: The conformation of ribosomal proteins free in solution has been studied by circular dichroism (CD) and the proteins have been shown to possess unique conformations. The ribosomal proteins which contain aromatic amino acid residues and disulfide bonds appear to have unique tertiary structures as shown by the presence of near-ultraviolet CD bands. Estimations of average secondary structure based on the far-ultraviolet CD spectra suggest the presence of significant amount of α helix and β structure. These unique secondary and tertiary structures are extensively disrupted by guanidinium chloride (GdmCl). Broad conformational transition profiles are observed for the denaturation by GdmCl in the concentration range of 0 to 3.5 M. Upon removal of the denaturant, the proteins from the 50S subunits are completely renatured, whereas partial renaturation of the proteins from the 30S subunits is observed. These unique conformations of ribosomal proteins are sensitive to ionic strength of the medium. The conformations of the proteins bound in the ribosomal particles in the functional medium and in reconstitution buffer have been approximated from CD studies by subtracting the RNA contribution from the CD spectrum of the intact ribosome and found to be different from the corresponding conformations free in solution. The change of conformation when the proteins are bound to the RNAs may have significant relevance to the interactions of the proteins and the RNAs in the assembly of functional ribosomal particles.

In an attempt to understand the molecular mechanism of the self-assembly process of the ribosome from its component proteins and RNAs, and particularly the role of proteins in this process, we have studied the conformation of ribosomal proteins under the conditions of reconstitution. Heretofore, few structural studies have been conducted under these reconstitution conditions (Traub & Nomura, 1969) in which the RNAs and the proteins are likely to assume their corresponding unique conformation necessary for the molecular recognition processes of protein–protein and protein–RNA interactions to form the ribosomal particles. Thus knowledge of the "preassembled" conformational state of the ribosomal proteins and the conformation they assume after reconstitution will provide some insights into the molecular mechanism of the assembly process and an understanding of the molecular forces responsible for the maintenance of the three-dimensional structure of the ribosome. In addition, such knowledge will enable us to elucidate the nature of conformational changes of the protein and RNA components during the functioning cycle of the ribosome in protein biosynthesis.

Several studies on the conformation of ribosomal proteins free in solution and bound in the ribosome have been published. However, conflicting results have been reported. Optical rotatory dispersion measurements on yeast ribosomes indicate that free ribosomal proteins have no α-helical structure but, when they are bound to the ribosome, the proteins acquire about 30% α-helical structure (McPhie & Gratzer, 1966). On the other hand, a similar study on *Escherichia coli* ribosome and its protein and RNA components suggests that the conformation of free ribosomal proteins is very similar to that within the ribosome and both are estimated to have about 25% α-helical structure (Sarkar et al., 1967). CD[1] measurements of individual proteins free in an acidic solution indicate an average of about 30% α helix (Dzionara, 1970). Cotter & Gratzer (1969a,b) have determined by infrared spectroscopy that free ribosomal proteins exist predominantly in β structure, but within the ribosome they possess little β structure. A more recent study (Lemieux et al., 1974) suggests that proteins isolated from the 30S subunit contain about 30% α-helical structure and 18% β structure in reconstitution buffer. The results of this investigation show that ribosomal proteins do possess unique secondary and tertiary structures which are sensitive to ionic strength of the medium and the presence of small amounts of guanidinium chloride. Furthermore, we have shown that the conformation of ribosomal proteins free in solution is different from that in the ribosomal particles.

Materials and Methods

E. coli Cells. Escherichia coli strain MRE 600 (RNase I$^-$) was grown under forced aeration at 37 °C in a Tryptone broth (1.3% tryptone, 0.7% NaCl) at pH 7.0. Cell suspensions in mid-log phase were chilled at 0 °C and harvested in a Sharpless continuous flow centrifuge. The cell paste was frozen and stored at −20 or −75 °C. Some recent MRE 600 *E. coli* cells were kindly provided to us by Professor Gary Craven at the University of Wisconsin.

Ribosome and Subunits. Ribosomes were isolated according to the procedure of Nomura & co-workers (Traub et al., 1971) with slight modification. To 100 g of frozen cell paste was added 60 mL of TMA buffer (10 mM Tris, 20 mM $MgCl_2$, 30 mM NH_4Cl, pH 7.6). The thawed cream was disrupted by a precooled French press operated at 9 to 12×10^3 psi. The lysate was diluted to approximately 300 mL with TMA buffer and the cell debris was removed by two successive centrifugations at 14 500 rpm for 30 min in a SS34 rotor of the Sorvall RC2-B centrifuge. The ribosomes were sedimented at 30 000 rpm with a type 30 rotor in the Beckman L5-65 ultracentrifuge for 6 h.

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[1] Abbreviations used: CD, circular dichroism; GdmCl (GdnCl in figures), guanidinium chloride; TP70, total proteins isolated from the 70S ribosome; TP30, total proteins isolated from the 30S ribosomal subunit; TP50, total proteins isolated from the 50S ribosomal subunit.

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Following resuspension in a minimum amount of TMA buffer and clarification by centrifugation for 10 min at 16 000 rpm in the SS34 rotor, the suspension was layered on 30 mL of a solution of 30% sucrose in TMA buffer. The ribosomes were sedimented at 30 000 rpm for 18 h in the type 30 rotor. The 18-h centrifugation and clarification steps were repeated. All operations were carried out at 2–5 °C. The final pellets were resuspended in TMA buffer and used immediately.

The subunits were isolated by centrifugation in a Beckman Ti-15 zonal rotor using a hyperbolic gradient from 7.4 to 38.0% sucrose according to the method of Eikenberry et al. (1970) with minor modifications. Sucrose solutions were treated with 0.1% diethylpyrocarbonate. The 800-mL sucrose gradient was pumped through the edge of a Ti-15 zonal rotor at a rate of 40 mL/min with a Beckman Model 114 gradient pump and followed by a 45% sucrose solution as cushion until the rotor was filled. Approximately 1.0 g of the 70S ribosome sample in 50 mL was dialyzed against a "30–50" buffer (10 mM Tris, 1 mM $MgCl_2$, 100 mM KCl, pH 7.4) in order to dissociate the subunits and was applied through the center of the rotor in a 100-mL 0–7.4% sucrose linear gradient using a peristaltic pump. This was followed by a 708-mL "30–50" buffer overlay. The separation was carried out at 22 000 rpm for 15 h at 4 °C. The rotor was unloaded by pumping a 60% solution of sucrose through the edge at a rate of 25 mL/min. Fractions of 15 mL were collected after the first 600 mL was discarded, and the absorbance of the fractions was determined at 260 nm. The 30S and 50S subunit fractions were pooled and sedimented by centrifugation with a Beckman type 65 rotor at 55 000 rpm at 4 °C for 24 and 16 h, respectively. Samples were resuspended in TMK_{360} buffer (20 mM Tris, 20 mM $MgCl_2$, 360 mM KCl, pH 7.6) and used immediately.

Activation of Ribosome. Activation of ribosome and its subunits was made by incubation of the ribosomal samples at 37–42 °C for 20 min before experiments (Zamir et al., 1974).

Characterization of Ribosome and Its Subunits. Sedimentation velocity analysis was used for characterization of the ribosomal particles. A Beckman Model E analytical ultracentrifuge equipped with Schlieren optics was employed.

Preparations of Ribosomal Proteins and RNAs. Total proteins from the 70S ribosome, 30S and 50S subunits, were extracted by the acetic acid method of Hardy et al. (1969). Ribosomal RNAs were extracted by the phenol-sodium dodecyl sulfate procedure (Traub et al., 1971).

Characterization of Ribosomal Proteins. Ribosomal proteins were characterized by two-dimensional polyacrylamide gel electrophoresis according to the method of Kaltschmidt & Wittmann (1970) with recent modifications by Howard & Traut (1974) and by Knopf et al. (1975).

Circular Dichroism. CD measurements were made with a JASCO J-20 spectropolarimeter. The temperature of the sample cell was controlled using a water-jacketed aluminum cell holder regulated by a Lauda K-2R water bath. The temperature of the sample was measured directly with a Model YSI 425 telethermometer. Absorbance of solutions was less than 1.5 at 260 nm. The results were expressed in terms of molar ellipticity, $[\theta]$, in units of deg cm^2 per dmol using the equation: $[\theta] = \theta M/dc$; where, θ is observed ellipticity in degrees, M is molecular weight, and d and c are the optical path length in decimeters and concentration in g/cm^3, respectively. The amount of secondary structure of ribosomal proteins was estimated according to the method of Chen et al. (1974), and for this purpose the CD spectra were also expressed as mean residue weight ellipticity, where a mean residue weight of 114 was used instead of the molecular weight. In order to obtain the optimal CD spectra, the path lengths of the CD cells for the near-UV regions 320–290 nm and 290–250 nm were 100 mm and 5 or 10 mm, respectively, and cells with path lengths of 0.5, 1, or 2 mm were used in the far-UV region 250–200 nm. The instrument was calibrated with *d*-camphor-10-sulfonic acid according to Cassim & Yang (1969).

The conformation of the ribosomal proteins bound in the ribosome was estimated by subtracting the RNA contribution from the far-UV CD spectrum of the corresponding ribosomal particle, as expressed in terms of molar ellipticity assuming the corresponding molecular weights of the mixtures of proteins. These spectra were then converted to mean residue weight ellipticity. An analysis of the secondary structure was carried out by the method of Yang and co-workers (Chen et al., 1974), using a BMD073 computer program (Biomedical Computer Programs, University of California Press, 1973) and IBM 370/145 computer.

Miscellaneous. The concentrations of 70S ribosome, 30S and 50S subunits, were determined spectrophotometrically with a Cary 118 spectrophotometer using $A_{260}^{1mg/mL} = 14.5$, 14.8, and 14.5, respectively (Hill et al., 1969a). The concentrations of the RNAs were determined using $A_{260}^{1mg/mL}$ of 22.3 (Stanley & Bock, 1965). Protein concentrations were determined in triplicate by the Lowry method as modified by Hartree (1972), using bovine serum albumin as a standard. Molecular weights for the 70S, 50S, 30S particles and their corresponding RNAs and total proteins used in the normalization of CD spectra were: 70S ribosome and the large and small subunits 2.65×10^6, 1.65×10^6, and 1.0×10^6, respectively (Hill et al., 1969a, 1970); for the total proteins, 0.906×10^6, 0.546×10^6, and 0.360×10^6, respectively, which were obtained by subtracting the mass of RNA from the mass of its corresponding intact particle.

pH measurements were obtained at room temperature with a Radiometer Model PHM64 research pH meter equipped with a combined glass electrode (GK 23226) and calibrated with pH 7.00 and pH 4.01 standard buffers. All water used was double distilled and deionized. Dialysis tubing was treated by boiling in 1% Na_2CO_3, 1 mM EDTA for 30 min and washed thoroughly with distilled water. Precautions were taken to avoid ribonuclease and protease contaminations.

Sequanal grade sodium dodecyl sulfate was purchased from Pierce Chemical Co. and Ultrapure GdmCl from Schwarz/Mann. All other chemicals used were reagent grade.

Results

Total ribosomal proteins from the 70S ribosome and those from the 30S and 50S subunits were studied and will be referred to as TP70, TP30, and TP50, respectively. The protein mixtures were characterized by two-dimensional gel electrophoresis (Knopf et al., 1975). The gel patterns are shown in Figure 1. They agree very well with those reported (Knopf et al., 1975; R. R. Traut, personal communication). We refer to the ribosomal proteins bound in the ribosomal particles as the bound proteins, and those free in solution as the free proteins. The results were obtained in two buffer media. The TMA buffer, referred to as functional buffer, is the medium normally used for functional assay of ribosomal activity. The TMK_{360} buffer is the medium used for the successful reconstitution of functional 30S subunit (Traub & Nomura, 1969) and the reconstitution of 5S RNA–protein complex (Horne & Erdmann, 1972; Gray et al., 1973), and is called the reconstitution buffer. All results were obtained at 37 ± 0.1 °C, unless specified otherwise.

Near-UV CD Spectra. The near-UV CD spectra for free TP30 and free TP50 are shown in Figure 2. The CD spectrum

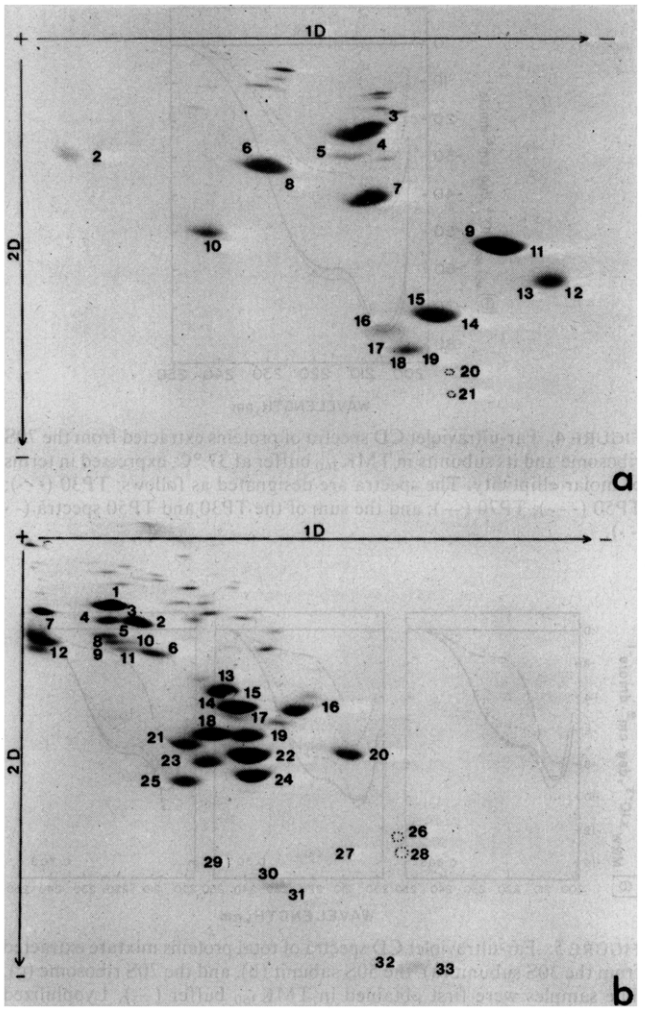

FIGURE 1: Two-dimensional gel electrophoresis pattern of proteins extracted from (a) 30S subunit and (b) 50S subunit. Approximately 250 μg of protein sample was separated on a discontinuous polyacrylamide gel electrophoresis system under acidic pH at 75 V for 6 to 7 h. The first dimension gel was polymerized into a gel slab and electrophoresis was carried out under acidic pH at 150 V for 6 to 9 h.

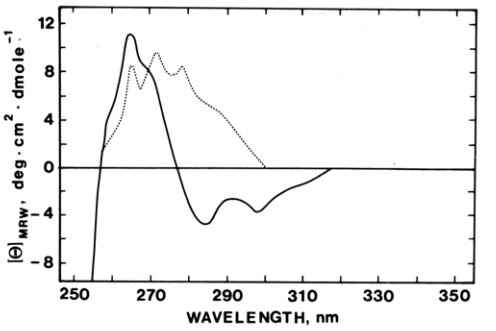

FIGURE 2: Near-UV CD spectra of proteins extracted from the ribosomal subunits. The protein concentration was 225 μg/mL for TP30 (···), and 350 μg/mL for TP50 (—).

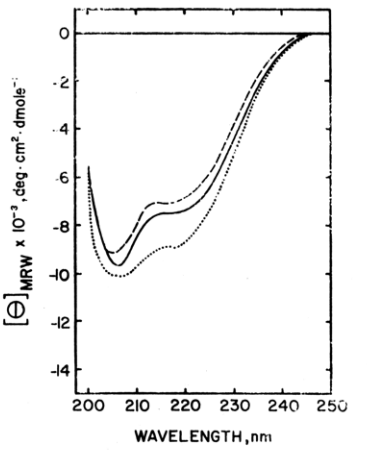

FIGURE 3: Far-UV CD spectra of proteins extracted from the ribosome and its subunits in TMK_{360} buffer at 37 °C. The spectra are designated as follows: TP30 (···), TP50 (- - -); TP70 (—). The protein concentration range was 0.07–0.2 mg/mL.

for free TP50 shows a negative trough at 299 nm, and another trough at 284 nm with a crossover at 276 nm. At lower wavelengths there is a relatively large peak at 264 nm with shoulders at about 270 nm and 258 nm and further crossover at 257 nm. The corresponding spectrum for free TP30 is quite different. It has three positive peaks at 265, 272, and 278 nm and a shoulder at 287 nm. The crossover is at 257 nm. In general, all the ellipticity bands are very small. The spectra could only be obtained with the use of 10-cm cells since the low solubility of ribosomal proteins did not permit the use of concentrations higher than about 1 mg/mL. Since CD bands in this spectral region arise from the tertiary conformational environment of the aromatic residues and also disulfide bonds, and ribosomal proteins have been known to have relatively few aromatic amino acid residues and disulfide bonds, the small magnitude of these ellipticity bands is not unexpected. Two consequences follow from these small ellipticity values. In several nonribosomal proteins containing aromatic amino acid residues and disulfide bonds, significant contributions of these optical activities to the far-UV CD ellipticity have raised questions on the quantitative estimations of secondary structure of these proteins. The small magnitude in near-UV CD presumably makes insignificant and negligible contributions to the estimation of secondary structure of ribosomal proteins. The other consequence is that the small near-UV ellipticity reinforces the contention that the near-UV CD spectra of ribosomal particles and ribosomal RNA–protein complexes are due almost completely to the RNA, and proteins contribute only negligibly to the CD spectra in this region.

Far-UV CD Spectra and Analyses of Secondary Structure. The far-UV CD spectra of free TP30, TP50, and TP70 were measured in reconstitution buffer and are shown in Figure 3. The general features characteristic of these spectra are the two negative CD bands at about 220 and 205–208 nm. A careful analysis of the secondary structure was carried out by the method of Chen et al. (1974) and the results are summarized in Table I. Free TP30 and TP50 contain both α helix and β structure. Free TP30 is shown to have more α-helical structure than free TP50 with 29% α helix as compared with 23% for free TP50. Both free TP30 and free TP50 contain approximately the same amount of β structure, i.e., 18%. Estimation of secondary structure of free TP70 results in values approximating an average of TP30 and TP50 with 24% α helix and 17% β structure. It is possible to express the CD spectra in terms of molar ellipticity instead of mean residue ellipticity and thus determine whether the CD spectrum of the sum of TP30 and TP50 is equal to that of TP70. Figure 4 shows that the CD spectra of TP30 and TP50 do closely approximate the CD spectrum of TP70 on a molar basis. It should be noted that the relative amount of secondary structure is estimated in terms of mean residue ellipticity. The far-UV CD spectra of the free proteins were also obtained in the functional medium (TMA buffer) which has lower ionic strength than the reconstitution

TABLE I: Estimation of Secondary Structure of Free and Bound Ribosomal Proteins in Reconstitution Buffer[a] at 37 °C.

	%		
	α helix	β structure	disordered
free TP30	29	18	53
bound TP30	34	7	59
free TP50	23	18	59
bound TP50	16	6	78
free TP70	24	17	59
bound TP70	24	6	70

[a] Reconstitution buffer (TMK_{360}) = 20 mM Tris, 20 mM $MgCl_2$, 360 mM KCl, pH 7.6.

TABLE II: Estimation of Secondary Structures of Free and Bound Ribosomal Proteins in a Functional Medium[a] at 37 °C.

	%		
	α helix	β structure	disordered
free TP30	22	15	63
bound TP30	29	5	66
free TP50	13	18	69
bound TP50	13	17	70

[a] Functional medium (TMA buffer): 10 mM Tris, 20 mM $MgCl_2$, 30 mM NH_4Cl, pH 7.6.

TABLE III: Estimation of Secondary Structure of Free Ribosomal Protein in 0.2 M of Residual Guanidinium Chloride[a] and after Complete Removal of the Denaturant by Dialysis.[b]

	%		
	α helix	β structure	disordered
TP30			
in 0.2 M GdmCl	17	19	64
after removal of GdmCl	26	13	61
TP50			
in 0.2 M GdmCl	19	19	62
after removal of GdmCl	22	19	59
TP70			
in 0.2 M GdmCl	20	16	64
after removal of GdmCl	24	10	66

[a] Lyophilzed sample solubilized in 6 M GdmCl and then diluted with TMK_{360} buffer to 0.2 M of residual GdmCl. [b] Residual GdmCl removed by exhaustive dialysis against TMK_{360} buffer.

buffer. The results of the conformational analyses are summarized in Table II and indicate that the α-helical content of "free" total proteins is dependent upon ionic strength, whereas the amount of β structure remains about the same. In both TP30 and TP50, the α-helical contents increase with higher ionic strength as in the case of reconstitution buffer.

Conformation of Ribosomal Proteins in Guanidinium Chloride. The far-UV CD spectra of TP30, TP50, and TP70 in the presence of 6 M GdmCl are shown by the broken curves in Figure 5. High concentrations of GdmCl are known to disrupt the conformation of many proteins and render them into random coiled polypeptides (e.g., Tanford, 1968; Wong & Tanford, 1973). The CD spectra of the ribosomal proteins in 6 M GdmCl are characteristic of proteins which have been unfolded into an extensively disordered conformation (Nozaki et al., 1974; Leach et al., 1974). The reversibility of this unfolding by GdmCl and particularly the question of whether low

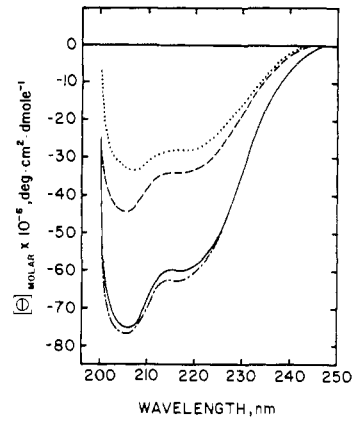

FIGURE 4: Far-ultraviolet CD spectra of proteins extracted from the 70S ribosome and its subunits in TMK_{360} buffer at 37 °C, expressed in terms of molar ellipticity. The spectra are designated as follows: TP30 (· · ·); TP50 (- - -); TP70 (—); and the sum of the TP30 and TP50 spectra (- · - ·).

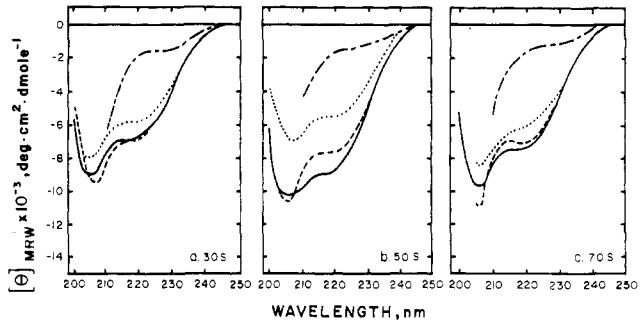

FIGURE 5: Far-ultraviolet CD spectra of total proteins mixture extracted from the 30S subunit (a), the 50S subunit (b), and the 70S ribosome (c). The samples were first obtained in TMK_{360} buffer (—). Lyophilized sample was solubilized in 6 M GdmCl (- · - ·) and diluted with TMK_{360} buffer to approximately 0.2 M residual GdmCl (· · ·) and then renatured by removing the residual GdmCl by dialysis against TMK_{360} buffer (- - -). All spectra were recorded at 37 °C.

concentrations of GdmCl affect the conformations of ribosomal proteins have been studied. This is necessitated by the wide use of the reagent for solubilizing ribosomal proteins for various types of studies. Ribosomal proteins in general have very low solubilities in various buffers. In many studies reported in the literature (Lemieux & Gerard, 1973; Lemieux et al., 1974; Rhode & Aune, 1975), this problem is circumvented by dissolving a small amount of lyophilized protein in aliquots of 6 M GdmCl and then diluting with buffer until the final concentration of GdmCl is approximately 0.2 M. Whether the conformation of ribosomal proteins which have been subjected to this treatment is similar to the conformation of ribosomal proteins which have never been lyophilized or treated with this denaturant has not been investigated. We have studied this problem by the following procedure: free ribosomal proteins were lyophilized, solubilized in a minimal amount of 6 M GdmCl, and diluted with TMK_{360} buffer. The resulting concentration of GdmCl is approximately 0.2 M which corresponds to the concentration range of residual GdmCl in the reported experiments (Lemieux & Gerard, 1973; Lemieux et al., 1974). The CD spectra of TP30, TP50, and TP70 were obtained and the results are shown by the dotted curves in Figure 5. The conformation of TP30 appears to be affected significantly, while TP50 is less affected. The relative amounts of secondary structure estimated from these spectra are listed in Table III. The α-helical content of the ribosomal

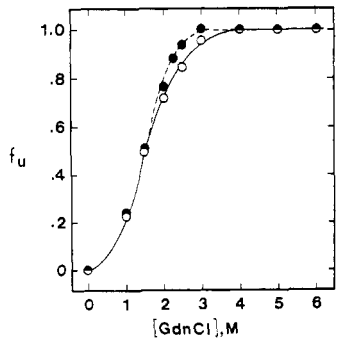

FIGURE 6: The denaturation profiles of ribosomal proteins in guanidinium chloride. f_u is the fraction of unfolding and is monitored by change in ellipticity at 222 nm, $[\theta]_{222nm}$. (Open circles) TP50; (closed circles) TP30. Temperature, 25 °C.

proteins which have been exposed to these conditions and remain in 0.2 M residual GdmCl is less than that of the native proteins. This difference is probably due to either the irreversible unfolding of some proteins caused by lyophilization and/or GdmCl treatment, or alternatively, the existence of a different conformational state at the residual GdmCl concentration. In order to ascertain whether or not this conformational state which exists in 0.2 M GdmCl can be renatured to the native state, each protein sample was dialyzed against a 500 times volume of TMK_{360} buffer overnight to remove the residual GdmCl and the CD spectra were then obtained. The results are shown by the dashed curves in Figure 5, and the conformational analyses are also listed in Table III. The conformation of TP50 appears to be almost completely reversible upon removal of residual GdmCl since the percentages of α-helical and β structures of these samples are nearly identical with the native conformation. The presence of 0.2 M residual GdmCl results in a conformational state of TP30 with decreasing α-helical contents, although the β structure is constant. Upon removal of this residual GdmCl by dialysis, the α-helical content is increased to almost the same as the native state, but the proteins now have decreasing amounts of β structure. Similar behavior is obtained for the TP70 as shown in Table III.

Conformational Transition. Many proteins which possess unique conformation in solution have been shown to undergo cooperative conformational transition by increasing concentrations of GdmCl and unfold into random coiled proteins at high concentrations of the reagent (e.g., Tanford, 1968; Wong & Tanford, 1973). The conformational change of TP30 and TP50 as a function of GdmCl concentrations has been studied by monitoring the change in ellipticity at 222 nm, $[\theta]_{222}$. Figure 6 shows the fraction of unfolding, f_u, by dividing the amount of change in $[\theta]_{222}$ from its native state by the total change in $[\theta]_{222}$, as a function of GdmCl concentrations. Both TP50 and TP30 undergo rather broad conformational transitions from 0 to 3.5 M GdmCl with the same midpoints of transition at about 1.5 M of GdmCl. Beyond the midpoint of transition, the transition curve for TP50 is wider, extending to 3.5 M GdmCl, while the unfolding of TP30 is completed at 3.0 M GdmCl.

Conformation of Ribosomal Proteins Bound in the Ribosome. Since the near-UV CD spectrum of RNA is extremely sensitive to its conformation (e.g., Brahms & Mommaerts, 1964), and it appears that the secondary structure of free RNA is about the same as that within the ribosome (Zubay & Wilkins, 1960; Klug et al., 1961; Cox, 1966; McPhie & Gratzer, 1966; Sarkar et al., 1967; Cotter et al., 1967; Bush & Scheraga, 1967; Tal, 1969; Miall & Walker, 1969; Cotter & Gratzer, 1969a; Thomas, 1969), the conformation of the

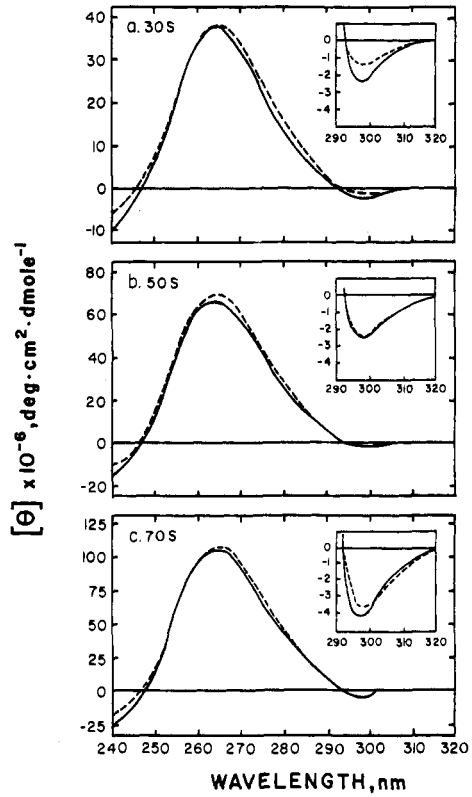

FIGURE 7: Near-UV CD spectra of ribosomal particles (—) and their corresponding RNAs free in solution (- - -). (a) The 30S subunit; (b) 50S subunit; and (c) 70S ribosome. Conditions: TMK_{360} buffer, 37 °C. Inset: enlarged portion of the CD spectra in 290–320 nm.

proteins in the ribosome may be estimated by subtracting the CD spectrum of the ribosomal RNA from the CD spectrum of the intact ribosomal particle. The near-UV CD spectra of the 30S, 50S, and 70S particles with their corresponding RNA and free and bound proteins under reconstitution conditions are shown in Figure 7. Although free ribosomal proteins have small aromatic CD bands, their contribution to the near-UV CD spectrum is so small that their ellipticity values are negligible in comparison with the CD spectrum of the RNA. The near-UV CD spectra of the ribosome and its subunits and their corresponding RNAs are characterized by a small trough at 297 nm and a large peak at 265 nm. The intact particles show crossovers at 292.5 and 247 nm, while the free rRNA shows crossovers at 292.5 and 246–247 nm. In general, the near-UV CD spectra of the ribosomal RNAs and those of the ribosome and its subunits are very similar. The magnitude of molar ellipticity for the RNAs at 265 nm is approximately 4% greater than that of the corresponding ribosomal particles. The magnitude of the 297 nm trough is identical for the 50S subunit and its RNAs; but for the 30S subunit, removal of the proteins results in a smaller magnitude. Similar quantitative results for the 30S and 50S ribosomal subunits and their corresponding RNAs have been independently reported by Cox & co-workers (1976). However, their results were obtained at 0.1 mM $MgCl_2$, 10 mM Na_3PO_4, 10 mM NH_4Cl, pH 7.

The far-UV CD spectra of the 30S subunit, 50S subunit, and the 70S ribosome, as well as their corresponding RNAs and free and bound ribosomal proteins in TMK_{360} at 37 °C are shown in Figure 8. The ribosomal particles exhibit two negative bands in the far-UV CD spectrum, one at 220–230 nm which is mainly due to ribosomal proteins and the other CD band at 208 nm which is greater than twice the magnitude of the shoulder at 222 nm and arises from both RNA and protein.

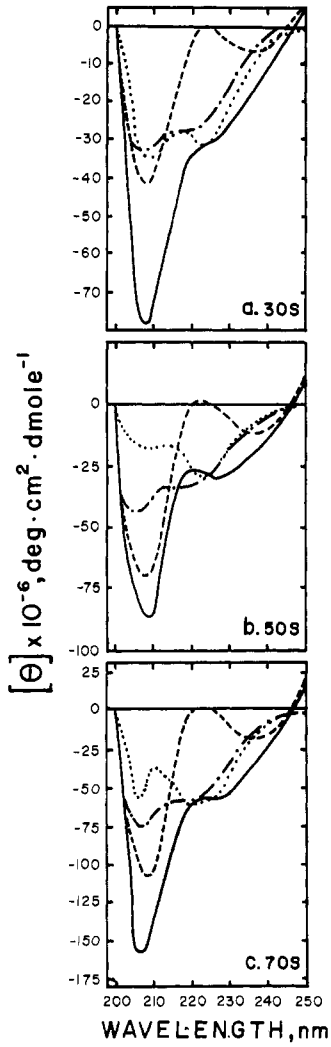

FIGURE 8: Far-UV CD spectra of ribosomal particles (—) and their corresponding RNAs (- - -) and proteins (· · · ·) free in solution. The far-UV CD spectra of the proteins bound in the ribosomal particle (· · ·) were calculated by subtracting the CD spectra of the RNAs from the CD spectrum of the corresponding intact ribosomal particles. Conditions: TMK_{360} buffer, 37 °C.

The results of the estimation of secondary structure corresponding to the free and bound ribosomal proteins under the condition of reconstitution are shown in Table I. These results indicate that TP30 assumes more α-helical structure, increasing from 29% to 34%, but less β structure decreasing from 18% to 7%, when it is bound to the 16S RNA in the 30S subunit. TP50 assumes a less ordered structure, the α and β structure decreasing from 23% to 16% and 18% to 6%, respectively. The β structure of TP70 likewise undergoes a significant decrease from 15% to 6%. The little change in the amount of α-helical content of TP70 is consistent with the averaging of the opposite trends seen in TP30 and TP50. Corresponding studies were also conducted in the functional medium, i.e., TMA buffer. The results of the conformational analyses of free and bound ribosomal proteins are summarized in Table II. The conformation of bound TP30 is sensitive to changes in ionic strength in a manner similar to that seen for the free proteins. However, no differences in the conformations are observed in functional medium for the free and bound TP50.

A limitation in this indirect method of obtaining the CD spectra of bound proteins by subtracting the CD spectrum of the RNA from that of the corresponding ribosomal particle should be mentioned. Normalization of CD spectra depends upon accurate knowledge of molecular weights and concentrations of the ribosomal particles and their component RNAs and proteins. Therefore, any small uncertainty in these parameters will be reflected in the normalized spectra. It is important to keep this in mind when comparing the CD spectra of a ribosomal particle and its RNA. Although we have estimated the amount of secondary structure for the bound ribosomal proteins, the values should be subjected to these uncertainties. What is important to note is that, in comparison with the free proteins, bound proteins also have significant amounts of secondary structure. Furthermore, the conformations of proteins free in solution are different from those bound in the ribosome.

Discussion

Ribosomal Proteins Have Unique Conformations Which Are Sensitive to Environmental Conditions. The results of this investigation suggest that ribosomal proteins free in solution have unique conformations which are sensitive to changes of environmental conditions. Several types of evidence lead to this conclusion. The existence of near-UV CD bands suggests that those ribosomal proteins possessing aromatic amino acid residues and disulfide bonds are likely to have unique tertiary structure since these CD bands arise from the rigid and asymmetric conformational environment imposed upon these chromophores. The far-UV CD spectra of ribosomal proteins are typical of proteins which contain unique conformations with well-defined secondary structure. The estimation of secondary structure of TP30 in reconstitution buffer is in good agreement with the average values (Lemieux et al., 1974) estimated for five individual 30S proteins in reconstitution buffer. They reported about 30% α helix and 18% β structure using a similar method of analysis. For TP50, the present results indicate that there is less α helix in free TP50 (23%) than in free TP30 (29%) but equivalent amount of β structure (18%). To our knowledge no other conformational study of TP50 in reconstitution buffer has been reported. Previous results on the conformational studies of ribosomal proteins are rather conflicting. Dzionara (1970) reported that both 30S and 50S ribosomal proteins have an average value of 30% α helix and little β structure based on CD studies. In this study the ribosomal proteins were in 10^{-3} M HCl. The ORD results of Sarkar et al. (1967) indicate that *E. coli* ribosomal proteins free in solution have approximately 25% α helix, whereas McPhie & Gratzer (1966) concluded that free proteins from yeast ribosomes contain no secondary structure. In another study, Cotter & Gratzer (1969a,b) determined by infrared spectroscopy that free ribosomal proteins exist predominantly as β structure. Whether the conditions used in these studies could cause the diverse results is difficult to assess. However, as shown by the present work, the conformation of ribosomal proteins free in solution is sensitive to ionic strength of the medium as well as small quantities of residual GdmCl used in solubilization of the lyophilized proteins.

Further evidence that free ribosomal proteins do possess unique conformations is indicated by their behavior toward the denaturant 6 M GdmCl. Guanidinium chloride is a reagent which has been used to disrupt the unique conformation of many nonribosomal proteins and render them into structureless random coiled polypeptides (e.g., Tanford, 1968; Wong & Tanford, 1973). Ribosomal proteins can also be transformed into random coiled proteins as shown by the fact that their CD spectra in 6 M GdmCl are similar to the CD spectra characteristic of random coiled or extensively disordered (Nozaki et

al., 1974; Leach et al., 1974). The fact that TP30 and TP50 free in solution possess CD spectra completely different from those of random coiled proteins shows that ribosomal proteins do not exist in a random coiled state and suggests that ribosomal proteins free in solution must assume unique well-defined conformation. The denaturation of TP50 appears to be completely reversible as shown by the studies in which the GdmCl was removed by dialysis. However, denaturation of free TP30 is only partially reversible. Thus, cation should be exercised in comparison of structural studies of the ribosome and its components particularly the 30S subunit, and in the interpretation of those studies which have not been conducted in functional medium or under the conditions of reconstitution. This is especially true of the ribosomal proteins whose conformations are sensitive to ionic strength as well as to the presence of small quantities of residual GdmCl used in solubilization of lyophilized proteins.

The Conformations of Ribosomal Proteins Bound in the Ribosome Are Different from Those Free in Solution. It appears that the net conformational changes which may occur in ribosomal RNA upon interactions with proteins to form the functional ribosomal particle are rather small and are of a subtle nature (Hochkeppel & Craven, 1977; Dunn & Wong, manuscript submitted for publication). The lack of significant changes in the CD spectra of RNA both bound and free in solution thus lends credence to the assumption used in obtaining the CD spectrum of the bound proteins by subtracting the CD spectra of the RNAs from the CD spectra of the corresponding ribosomal particles. The results obtained here suggest that ribosomal proteins undergo conformational changes upon assembly into ribosomal particles. Although similar studies using optical rotatory dispersion have been performed by McPhie & Gratzer (1966) and by Sarkar et al. (1967) under similar buffer conditions, their results are conflicting and are different from ours. Sarkar et al. (1967) reported that free ribosomal proteins (TP70) and bound ribosomal proteins have 25% α helix with no β structure, while McPhie & Gratzer (1966) concluded that free ribosomal proteins have no ordered structure but possess 32% α helix when bound in the ribosome. Using infrared spectroscopy Cotter & Gratzer (1969a,b) suggested that there is a significant amount of β structure in the free ribosomal proteins, but little β structure for the proteins bound in the ribosome. They also reported little α helix in either the free or the bound proteins. Our conclusion is that ribosomal proteins free in solution contain both α helix and β structure and these secondary structures undergo significant changes upon interaction with their respective RNAs to form the ribosomal particle.

Significance of Protein Conformations in Ribosome Assembly. The existence of unique conformational states in free ribosomal proteins and the susceptibility of these unique conformations to transformation into other unique conformational states by varying the ionic strength of the medium as required in the reconstitution of ribosome suggest the importance of protein conformation in the molecular recognitions of this assembly process. The high ionic strength requirements found by Nomura & co-workers can be explained by the present finding that many proteins are transformed into different unique conformation states and that the new conformational state at high ionic strength is the exact three-dimensional structure required for interaction with other proteins, and/or with the RNA, to assemble into a functional structure of the ribosome. The net conformational changes of proteins upon assembly of the ribosome may be attributed to the creation of new binding sites in the intermediate assembly particles for the further binding of subsequent proteins.

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Inhibition of Transfer RNA Function by Replacement of Uridine and Uridine-Derived Nucleosides with 5-Fluorouridine†

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ABSTRACT: As part of a study of the functional significance of minor nucleotides, the protein synthetic activity of *Escherichia coli* tRNA, with most of its uridine and uridine-derived residues replaced by 5-fluorouridine, was examined. Although the modified tRNA could be charged with all amino acids, the rate of aminoacylation with lysine was only 3–7% of normal. Similar, though less marked, inhibitory effects were noted with aspartate, glutamate, glutamine, and histidine; 13 other amino acids were accepted at normal rates. Incorporation of 5-fluorouridine decreased the affinity of lysine tRNA for its synthetase, as indicated by a fivefold increase in the apparent K_M. The aminoacylation of analogue-containing tRNA with lysine was incomplete and increased with increasing concentrations of synthetase; at lower enzyme levels the extent of charging was proportional to enzyme concentration. Irreversible inactivation of synthetase or degradation of tRNA was ruled out. The rates of enzymatic and nonenzymatic deacylation of lysyl-tRNA were similar for normal and fluorouridine-substituted tRNA, indicating that incomplete charging of the analogue-containing tRNA with lysine is due to the lower rate of aminoacylation. Three peaks of lysine acceptor activity, isolated from 5-fluorouridine-containing tRNA by benzoylated diethylaminoethyl-cellulose chromatography, each exhibited the low rate and extent of lysine acceptance observed with unfractionated tRNA. Analogue-substituted lysine tRNA is able to stimulate only low levels of polypeptide synthesis in a tRNA-dependent cell-free system directed by synthetic polynucleotides or phage f2 RNA. Ribosome-binding studies showed that fluorouridine-containing lysyl- and glutamyl-tRNA were bound less efficiently than normal aminoacyl-tRNA in response to poly(A), poly(A,C), or poly(A,G). It is suggested that the reduction in the initial rate of charging and of ribosome binding observed with several tRNAs is due to fluorouridine-induced changes in the anticodon region of these molecules.

E scherichia coli transfer RNA which has 90–95% of its uridine and uridine-derived nucleosides replaced by 5-fluorouridine (Horowitz and Chargaff, 1958; Horowitz and Huntington, 1967; Lowrie and Bergquist, 1958; Johnson et al., 1969; Geige et al., 1969a; Kaiser et al., 1969; Kaiser, 1969a, 1972; Horowitz et al., 1974) has been used to probe the relationship between tRNA structure and function and to study the biological role of modified nucleosides (Horowitz et al., 1974, 1977; Ofengand et al., 1974). Previous results from this and other laboratories have indicated that highly substituted, unfractionated tRNA is able to accept all amino acids (Horowitz and Huntington, 1967; Lowrie and Bergquist, 1968; Johnson et al., 1969; Kaiser, 1969b; Geige et al., 1969b), and more detailed investigations with purified FUrd[1]-containing *E. coli* $tRNA^{Val}_1$ have shown that the rate of aminoacylation of this tRNA is unimpaired (Horowitz et al., 1974). FUrd-substituted $tRNA^{Val}_1$ is also able to function as well as control tRNA in all the subsequent steps of protein synthesis. Ternary complex formation with EFTu-GTP, EFTu-dependent binding to the ribosomal A site, nonenzymatic binding to the

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[1] Abbreviations used are: BD-cellulose, benzoylated diethylaminoethylcellulose; FUrd, 5-fluorouridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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