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Properties of *Escherichia coli* 16S Ribosomal Ribonucleic Acid Treated with 4,5′,8-Trimethylpsoralen and Light†

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ABSTRACT: 16S rRNA reacted with the furocoumarin 4,5′,8-trimethylpsoralen (trioxsalen) and 360-nm light showed a number of chemical and physical differences from untreated RNA. After extensive irradiation, five molecules of trioxsalen were bound per molecule of RNA. The trioxsalen-treated RNA had an altered ultraviolet absorption spectrum and a distinctive fluorescence emission spectrum. The modified RNA was significantly more resistant to T_1 ribonuclease digestion than was control RNA. Treated RNA, when mixed with purified ribosomal proteins, was not functional in the in vitro reconstitution of 30S subunits and yielded more slowly sedimenting particles which were inactive in protein synthesis assays. By contrast, 16S rRNA within the 30S subunit structure did not exhibit these changes when reacted with the same dose of trioxsalen and light, suggesting that the ribosomal proteins were effective in protecting the RNA from interaction with the drug.

Specific secondary and tertiary structures of rRNA are important in the formation, structure, and function of ribosomes (Zimmermann, 1974; Kurland, 1977). In particular, the 16S rRNA from *Escherichia coli* has been described as a flexible molecule whose conformation in solution can be altered by the ionic environment (Cox & Littauer, 1962), temperature (Schulte et al., 1974), or ribosomal proteins (Sypherd, 1971; Seals & Champney, 1976; Hochkeppel & Craven, 1976, 1977).

The complete in vitro reassembly of 30S subunits (reconstitution) takes place after a substantial conformational change of the 16S RNA has occurred (Held & Nomura, 1973). At low temperatures a protein-deficient reconstitution intermediate (RI) particle is formed. Heat activation of this 21S RI particle produces a more compacted 26S particle (RI*), with the same protein composition. This RI to RI* transition has recently been interpreted as an open to closed transformation of the tertiary structure of the 16S rRNA (Hochkeppel & Craven, 1977).

Little work has been done regarding other possible changes in the secondary or tertiary structure of the 16S RNA during the in vitro assembly process (Bollen et al., 1970). The identification of specific 16S RNA structural states during assembly is made difficult by the observation that these states are generally maintained only in the presence of certain ribosomal proteins. Deproteinization of a specific ribonucleoprotein intermediate can lead to a randomization of the particular RNA structural state.

Psoralen derivatives have been used to stabilize nucleic acid structures. These furocoumarins intercalate into nucleic acids, forming single-strand monoadducts and interstrand cross-links in single- or double-stranded DNA and RNA molecules (Rodighiero et al., 1970; Cole, 1971; Isaacs et al., 1977). The equilibrium binding constant for psoralen–nucleic acid complex formation is affected by such factors as the extent of secondary structure present in the nucleic acid, the type of psoralen derivative used, and the ionic strength and temperature of the solution (Dall'Acqua & Rodighiero, 1966; Dall'Acqua et al., 1969). The latter variables seem mainly to affect the structural state of the nucleic acid but not the photochemical reaction itself. Increasing the ionic strength of a nucleic acid solution (Shen & Hearst, 1976) or decreasing the temperature (Dall'Acqua et al., 1969) will result in a more extensive secondary (and tertiary) structure in the molecule. Consequently, the formation of molecular complexes between psoralens and nucleic acids would be expected to increase under these conditions (Rodighiero et al., 1970). For a particular nucleic acid, under fixed conditions of temperature, ionic strength, and light dose, the extent of drug photobinding should reflect the structural status of the nucleic acid in its specific environment. Thus, psoralens can be used not only to stabilize nucleic acid structure but also as a probe for detecting differences in the nucleic acid configuration in specific environments.

Recently, the stabilization of both double- and single-stranded DNAs (Cech & Pardue, 1976; Shen & Hearst, 1976) and RNAs (Hearst & Thiry, 1977; Issacs et al., 1977) from several sources has been reported. These observations, in combination with the demonstrated binding of 8-methoxypsoralen to *E. coli* transfer RNA (Ou & Song, 1978), suggested the possibility of using trioxsalen[1] to stabilize 16S rRNA secondary structure. In this communication, we describe the use of trioxsalen treatment as a technique for fixing the structure of 16S rRNA, in the presence and absence of 30S ribosomal proteins. The relative nonreactivity of the RNA

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[1] Abbreviations used: trioxsalen, 4,5′,8-trimethylpsoralen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; A_{260}, absorbance at 260 nm of a 1-mL solution in a 1-cm light path.

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within the ribosomal subunit, compared to protein-free RNA, has led us to conclude that the macromolecule is significantly more protected when it is complexed with ribosomal proteins.

Materials and Methods

Isolation of Ribosomal Subunits, Proteins, and RNA. 70S ribosomes from *E. coli* strain Q[13] (Reiner, 1969) were isolated and washed with 1 M NH_4Cl as previously described (Kushner et al., 1977). Subunits were separated by zonal centrifugation (19000 rpm for 17 h in a Beckman J21B centrifuge) through a hyperbolic sucrose gradient (Eikenberry et al., 1970). 30S and 50S subunits were recovered by ethanol precipitation of the gradient fractions.

30S ribosomal proteins were extracted by the LiCl–urea method (Traub & Nomura, 1969) and further purified by an acetic acid extraction and acetone precipitation step (Barritault et al., 1976). The proteins were stored at −70 °C in buffer III (10 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 1 M KCl, and 6 mM mercaptoethanol; Traub & Nomura, 1969), and the concentration was determined by the Lowry assay (Lowry et al., 1951).

A portion of the isolated ribosomal proteins was reductively methylated with [^{14}C]formaldehyde (42 mCi/mmol; New England Nuclear) in the presence of sodium cyanoborohydride (Aldrich Chemical Co.) by following the procedure of Dottavio-Martin & Revel (1978). The labeling was conducted with the proteins at 3.5 mg/mL in buffer III with the Tris-HCl replaced by 10 mM potassium phosphate, pH 7.2. After a 1-h incubation at 37 °C, with two additions of $NaBH_3CN$ (10 mg/mL), the protein solution was dialyzed extensively into buffer III. The protein specific activity was 2×10^6 cpm/mg.

16S rRNA was isolated from the 30S subunits by phenol extraction as previously described (Champney & Sypherd, 1970). After ethanol precipitation, the RNA was dissolved in buffer 0 (buffer III without KCl), and the concentration was determined from the absorbance at 260 nm (1 A_{260} unit/mL = 45 μg/mL RNA; Kurland, 1960). Homogeneity of the RNA was determined by polyacrylamide gel electrophoresis in the presence of formamide as described below.

Treatment of 16S RNA with Trioxsalen and Light. rRNA (22 μg/mL in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA) or 30S subunits (33 μg/mL) in either inactivation buffer [10 mM Tris-HCl, pH 7.6, and 0.5 mM $Mg(OAc)_2$] or activation buffer [20 mM Tris-HCl, pH 7.6, 20 mM $Mg(OAc)_2$, 0.2 M NH_4Cl, and 3 mM mercaptoethanol] were irradiated with and without trioxsalen. 4,5′,-8-Trimethylpsoralen (from Paul B. Elder Co.; recrystallized twice from chloroform and hexane) was dissolved in ethanol at 1 mg/mL (stock trioxsalen solution). Sufficient amounts of the stock trioxsalen were added to the RNA or subunit solutions to give a ratio of one molecule of drug per three RNA nucleotides. The mixture was kept at 0 °C in the dark for 30 min to permit intercalation. The mixture was stirred in a plastic petri dish at 4 °C during the irradiation. The near-ultraviolet light source was a General Electric Black Light bulb (F15T8/BLB) with an output intensity at 360 nm of 3 mW/cm^2 (as measured with a black ray ultraviolet meter, Model J221). Following each hour of irradiation, additional trioxsalen was added (1 μg/mL solution being treated). After irradiation, unreacted trioxsalen and other photochemical byproducts were removed from the the RNA by two extractions with chloroform–isoamyl alcohol (24:1) in the presence of 0.18 M NaCl (Isaacs et al., 1977). After an additional phenol extraction and overnight dialysis (in the Tris-HCl and EDTA buffer), the RNA was recovered by ethanol precipitation and stored in buffer 0 at −70 °C. This extraction procedure was necessary to remove all the unbound trioxsalen. 16S RNA from treated 30S subunits was extracted twice with phenol (in the presence of excess EDTA) and then further purified as described above.

[^{3}H]Trioxsalen Purification and Binding. [^{3}H]Trioxsalen was prepared by catalytic isotope exchange by the New England Nuclear Co. The radioactive compound was purified by thin-layer chromatography on silica gel plates in $CHCl_3$–methanol (98:2) as described (Isaacs et al., 1977). The purified [^{3}H]trioxsalen had the same absorption spectrum as the unlabeled drug and had a specific activity of 1.1×10^5 cpm/μg. It was stored at −70 °C in ethanol and used for RNA or 30S subunit binding exactly as described for the nonradioactive form. 16S RNA containing bound [^{3}H]trioxsalen was purified as described above, and samples were counted in 5 mL of a toluene–Triton X-100–2,5-diphenyloxazole (PPO) liquid scintillation cocktail (Yoakum & Cole, 1978).

Measurements of Absorbance and Fluorescence Spectra. Ultraviolet absorbance spectral determinations were made on 10–50 μg of RNA per mL of water in a Beckman Model 25 spectrophotometer. Fluorescence spectra were measured in an Aminco-Bowman spectrophotofluorometer with 5-mm excitation and emission slits by using samples of 20 μg of RNA in 1.5 mL of water.

Polyacrylamide Gel Electrophoresis of RNA. RNA samples were examined by electrophoresis in 3.5% polyacrylamide gels containing phosphate-buffered 99% formamide by the method of Duesberg & Vogt (1973). The gels were polymerized in plexiglass tubes (0.6 × 7 cm), and the samples were run at 80 V for 3–4 h until the bromophenol blue tracking dye was near the bottom of the tube. The RNA was stained by soaking the gel in a 1 μg/mL solution of ethidium bromide for 5–12 h. Under these conditions as little as 1 μg of 16S RNA could be easily detected. The stained RNA was visualized by examining the gels on a UV light box, and the gels were photographed through a red filter (Wrattan 23A) on Polaroid P/N 55 film.

T_1 Ribonuclease Digestion of RNA. The susceptibility to ribonuclease digestion was examined by incubation of 5-μg RNA samples with the T_1 enzyme (0.1 μg) in 100 μL of buffer 0 at 0 °C. To measure the kinetics of digestion, the incubation was terminated after various times by the addition of 1 mL of absolute ethanol. The samples were precipitated at −70 °C overnight, and the RNA was recovered by centrifugation at 10000 rpm for 3–4 h at −10 °C. The RNA pellets were vacuum dried, and the products were analyzed by polyacrylamide gel electrophoresis in formamide as described. The relative amount of 16S RNA remaining was estimated from the area of the 16S region of the stained gel, as determined by scanning the photographic negative in a spectrophotometer at 600 nm.

30S Subunit Reconstitution. The reconstitution of 30S subunits from 16S RNA and total 30S proteins was performed as previously described (Traub & Nomura, 1969) with some modifications. Generally, 25–50 μg of 16S RNA in 135 μL of buffer 0 was incubated at 40 °C for 10 min, then 3.5 equiv of 30S protein in 68 μL of buffer III was added, and the incubation was continued for an additional 20 or 45 min. (One protein equivalent is the amount of protein derived from 1 A_{260} unit of 30S subunits).

To recover reconstituted subunits, the incubation mixture was cooled and applied to a 5–20% sucrose gradient made in S buffer (10 mM Tris-HCl, pH 7.6, 0.5 mM $Mg(OAc)_2$, and 50 mM NH_4Cl). The samples were centrifuged at 45000 rpm for 185 min at 2 °C in a SW 50.1 rotor, and the subunits were

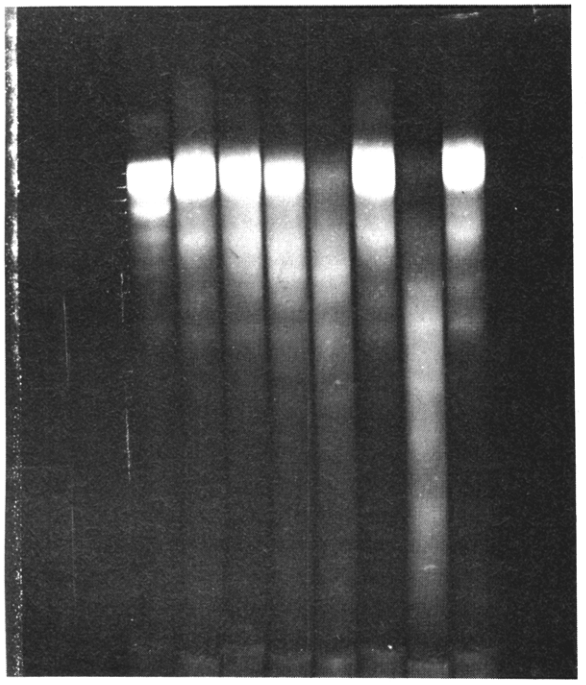

FIGURE 1: Polyacrylamide gel electrophoresis of 16S rRNA and T_1 ribonuclease digestion products. The electrophoresis in 3.5% gels containing 99% formamide was conducted as described under Materials and Methods. The samples were (a) 5 μg of native RNA; (b)–(e) 5-μg samples of trioxsalen-treated RNA (22×10^4 J/m^2) digested with T_1 ribonuclease for 0 (b), 15 (c), 30 (d), or 60 min (e); (f) 5 μg of trioxsalen-treated RNA incubated at 40 °C for 55 min; (g) 5 μg of trioxsalen-treated RNA reisolated after reconstitution with 30S proteins (45 min at 40 °C); and (h) same as (g) except the reconstitution mixture was kept at 0 °C.

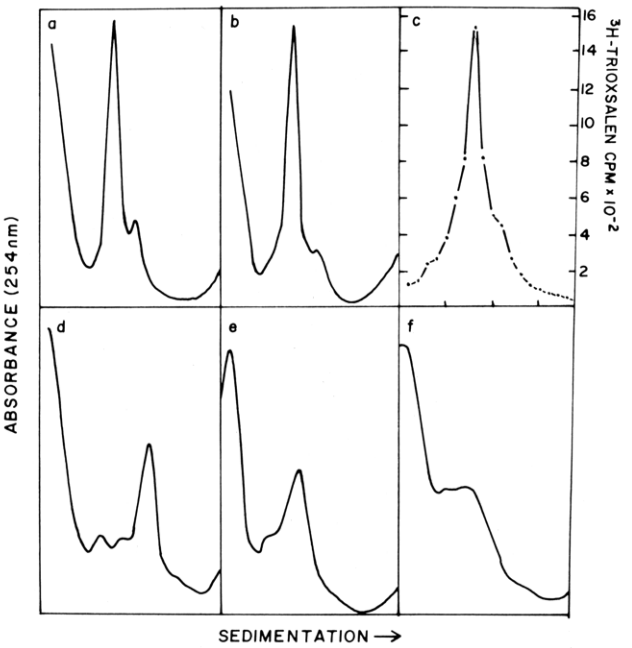

FIGURE 2: Sucrose density gradient profiles of 16S RNA and 30S subunit reconstitution products. The centrifugation was conducted as described under Materials and Methods. (a) Native 16S RNA. (b) Trioxsalen-treated RNA (22×10^4 J/m^2). (c) [^{3}H]Trioxsalen-treated RNA (44×10^4 J/m^2). Twenty fractions were collected for radioactivity measurements as described under Materials and Methods. The absorbance profile was the same as that in (b). (d) Particles produced by reconstitution of native 16S RNA with 30S proteins, incubated 45 min at 40 °C. (e) Particles produced by reconstitution of trioxsalen-treated 16S RNA [as in (b)] with 30S proteins, incubated 20 min at 40 °C. (f) Particles produced by reconstitution of trioxsalen-treated 16S RNA [as in (b)] with 30S proteins, incubated 45 min at 40 °C.

collected by displacing the gradient through an Isco Gradient Fractionating Device connected to a UV monitor and chart recorder. The RNA contained in the particles collected from the gradient was purified by phenol extraction as described and analyzed for homogeneity by electrophoresis in formamide-containing polyacrylamide gels and by sedimentation in sucrose gradients as described above.

Reconstitution with ^{14}C-labeled 30S proteins was conducted as described above. Radioactive particles recovered from the sucrose gradients were mixed with 25 A_{260} units of native 30S subunits and then dialized for 3–5 h in a 10 mM Tris-HCl and 10 mM $Mg(OAc)_2$ buffer, pH 7.6. After dialysis, the samples were lyopholized and dissolved in 1.5 mL of dialysis buffer. The ribosomal proteins were extracted with acetic acid and precipitated with acetone as described above. The proteins were separated by two-dimensional gel electrophoresis as described (Howard & Traut, 1973; Champney, 1977). Fluorographic detection of the radioactive proteins was performed as described by Bonner & Laskey (1974). For quantitative analysis the individual stained proteins were cut from the dried gel and counted directly in a 2.5% PPO–toluene solution.

In Vitro Protein Synthesis Assays. The assay methods for measuring the poly(uridylic acid)-directed formation of poly(phenylalanine) have been previously described (Traub et al., 1971; Kushner et al., 1977). The reaction mixtures contained 0.5 A_{260} units of native or reconstituted particles in the reconstitution buffer (buffer III containing 0.35 M KCl), in addition to 1 A_{260} unit of 50S subunits and other components as indicated above. The mixture was incubated at 37 °C for 45 min, and the amount of [^{14}C]phenylalanine present as 5% trichloroacetic acid precipitable radioactive material was collected on glass-fiber filters and counted in a liquid scintillation counter.

Subunit Reassociation Assays. The reassociation of native or reconstituted particles with 50S subunits to give 70S ribosomes was promoted by mixing 0.5–1 A_{260} units of particles with twice the amount of 50S subunits. The mixture was incubated in R buffer [S buffer containing 20 mM $Mg(OAc)_2$] at 37 °C for 30 min. The yield of 70S ribosomes was measured by sedimenting the mixture on a 5–20% sucrose gradient in R buffer at 35000 rpm for 135 min in an SW 50.1 rotor, followed by fractionation of the gradient through the Isco apparatus as described above The areas of the 70S and subunit peaks were measured on the recorder tracing.

Results

Homogeneity of Trioxsalen-Treated 16S RNA. It has been reported that singlet oxygen atoms, generated by the irradiation of psoralens, can cause fragmentation of nucleic acids by photooxidation (Ou & Song, 1978). No degradation was observed when 16S rRNA was treated with trioxsalen as described under Materials and Methods. Under denaturing conditions (electrophoresis in 99% formamide containing polyacrylamide gels), the majority of the RNA migrated as a single species (Figure 1b) in a slightly broader band than that of the control RNA (Figure 1a). In a 5–20% sucrose gradient the treated RNA sedimented as a single component with the same sedimentation properties as untreated RNA samples (Figure 2a,b).

Binding of [^{3}H]Trioxsalen. The amount of trioxsalen bound to the RNA under these conditions was determined by the use of ^{3}H-labeled drug. Figure 3 shows a saturation curve with increasing light dose for the binding of labeled trioxsalen. Five

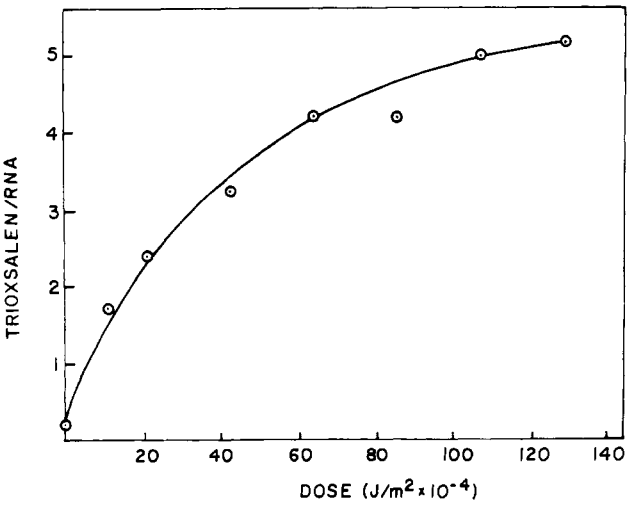

FIGURE 3: Saturation curve for the binding of [^{3}H]trioxsalen to 16S RNA. From the amount of radioactivity present in purified 16S RNA reacted with [^{3}H]trioxsalen at different doses of light, the number of bound trioxsalen molecules was calculated. At saturation (12.5 × 10^5 J/m^2) there was 9.93 × 10^3 cpm/A_{260} of purified RNA, corresponding to five molecules of trioxsalen per molecule of RNA. The results are the average of two experiments.

molecules of trioxsalen were bound per molecule of 16S RNA at the highest light dose. The half-saturation value was 23 × 10^4 J/m^2. Sedimentation analysis of [^{3}H]trioxsalen-labeled RNA revealed that all of the radioactivity cosedimented with the RNA peak (Figure 2c).

Absorbance and Fluorescence Spectra. 16S RNA treated with trioxsalen was significantly altered in both its ultraviolet absorbance and fluorescence spectra. An increase in the 235-nm ultraviolet absorbance and a 3–5 nm blue shift in the 260-nm absorbance maximum were consistently observed (Figure 4). A characteristic fluorescence emission peak at 395 nm was observed when treated samples were excited at 315 nm (Figure 5d–f). The magnitude of this fluorescence peak was dose dependent (inset to Figure 5) and did not appear in untreated control RNA samples (Figure 5a–c).

T_1 Ribonuclease Digestion. The rate of loss of 16S RNA after T_1 ribonuclease digestion for various lengths of time represents the relative susceptibility of the molecule to the enzymatic action under a given set of conditions. The capacity of RNA fragments to form hydrogen bonds with each other could lead to inaccurate estimates of the rate of degradation. To avoid this complication, digested RNA samples were examined under denaturing conditions where breakage of even a single phosphodiester bond is reflected in the loss of 16S RNA in the denaturing gels (Figure 1b–e). The rate of loss of intact 16S RNA was expressed as the half-life ($t_{1/2}$) of the first-order kinetic segment of the RNA decay curve.

Under the chosen digestion conditions, native and control RNA samples were degraded with a $t_{1/2}$ of about 12 min (Figure 6). Trioxsalen-treated 16S RNA showed a dose-dependent increase in the $t_{1/2}$ for digestion. The increase in relative $t_{1/2}$ for trioxsalen-treated samples indicated sigmoidal kinetics as shown in the inset to Figure 6.

Reconstitution of 30S Subunits. The reassembly of 30S subunits from their constituent proteins and RNA was used as an assay for the effects of trioxsalen on rRNA structure and function. Control and treated RNA preparations were analyzed for 30S subunit formation by sucrose gradient sedimentation of reconstitution mixtures and for their functional activity in protein synthesis assays. Control RNA samples gave reconstituted particles which sedimented like native 30S subunits (Figure 2d) and which were active in protein synthesis (Table I). Reconstitution experiments with trioxsalen-treated RNA yielded slowly sedimenting particles with a sedimentation coefficient of about 22 S (Figure 2e).

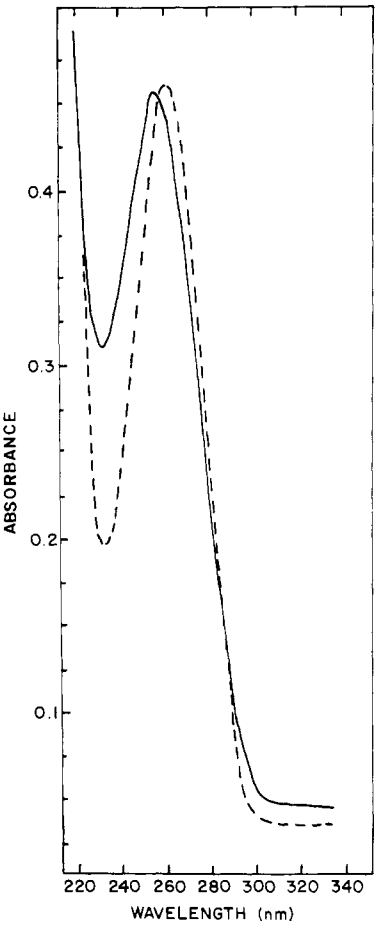

FIGURE 4: Ultraviolet absorption spectra of 16S RNA. The spectra of native RNA (- - -) and trioxsalen-treated RNA (11 × 10^5 J/m^2) (—) were recorded as described under Materials and Methods.

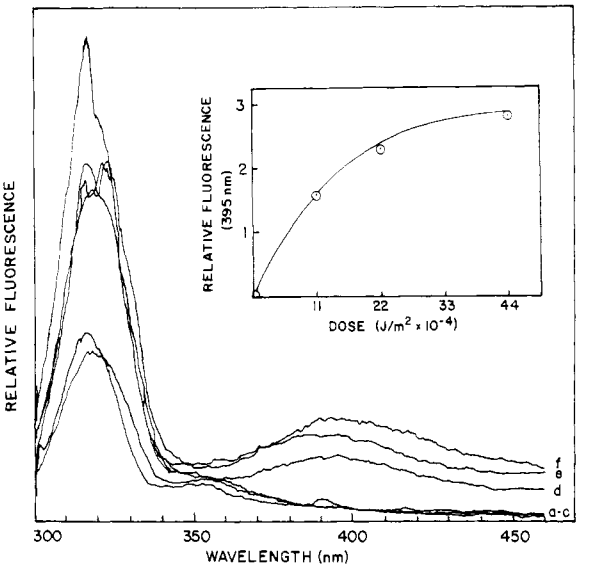

FIGURE 5: Fluorescence emission spectra of control and trioxsalen-treated 16S RNA. The excitation wavelength was 315 nm. The spectra shown are for samples (a) untreated, (b) trioxsalen-treated without irradiation, (c) irradiated (44 × 10^4 J/m^2) without trioxsalen, (d) trioxsalen-treated and irradiated at a dose of 11 × 10^4 J/m^2, (e) trioxsalen-treated and irradiated at a dose of 22 × 10^4 J/m^2, and (f) trioxsalen-treated and irradiated at a dose of 44 × 10^4 J/m^2. Inset: the dose dependence of the 395-nm emission maximum is indicated for the trioxsalen-treated samples.

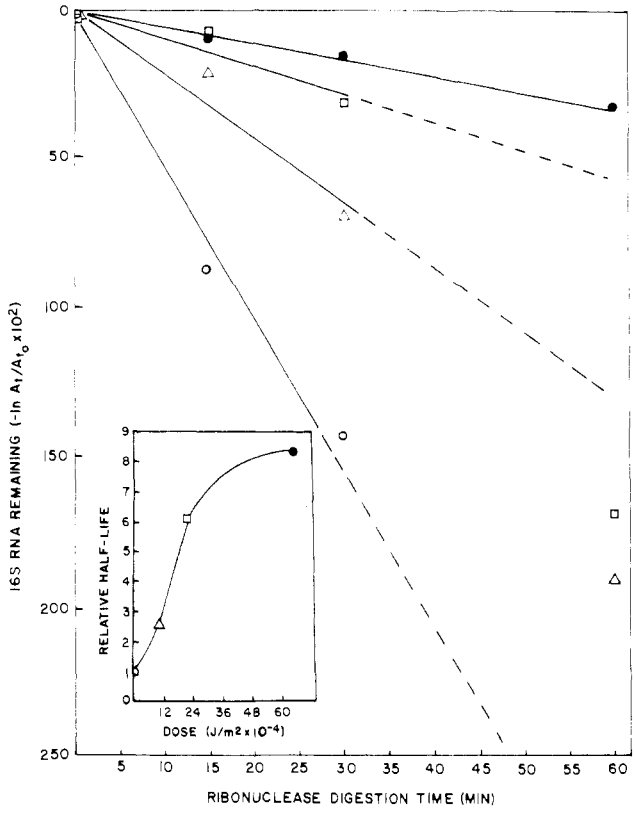

FIGURE 6: The kinetics of T_1 ribonuclease digestion of control and trioxsalen-treated 16S RNA samples. The extent of degradation of the 16S RNA was measured by polyacrylamide gel electrophoresis as described under Materials and Methods. The lines represent the amount of 16S RNA remaining after digestion for the indicated time, by using samples of control RNA (○) and trioxsalen-treated RNA at doses of 11 × 10^4 J/m^2 (△), 22 × 10^4 J/m^2 (□), and 65 × 10^4 J/m^2 (●). The amount of 16S RNA remaining was calculated by assuming a first-order decay for the initial rates. Inset: the dose dependence of the relative increase of the 16S RNA half-life. The half-life was calculated from the slope of the solid lines by assuming a first-order decay ($t_{1/2}$ = −ln 2/slope). The relative half-life is the ratio of the $t_{1/2}$ of the trioxsalen-treated samples to the $t_{1/2}$ of control RNA.

These particles were unstable (Figure 2f), and the RNA was degraded (by a ribonuclease in the 30S protein preparation) upon prolonged incubation in the reconstitution mixture (Figure 1g). No degradation was observed if the treated RNA was incubated without proteins (Figure 1f) or if the RNA and protein mixture was maintained at 0 °C (Figure 1h). In addition, the 22S particles were not active in the protein synthesis assay (Table I).

The protein composition of the reconstituted particles formed with native and trioxsalen-treated RNAs was examined by two-dimensional polyacrylamide gel electrophoresis of the isolated protein mixtures. The relative amount of each 30S protein present was determined by comparing the radioactivity present in an individual protein to the total present in all proteins isolated from the gel. The results are given in Table II for both types of particles (30 S and 22 S). Reconstitution with trioxsalen-treated RNA gave particles containing all of the proteins found in reconstituted 30S subunits. Reduced amounts of proteins S1, S2, S3, S10, S14, and S16 were present in the 22S particles, compared to the amounts found in the 30S subunits.

The particles produced under reconstitution conditions with control 16S RNA were examined for their ability to associate with 50S subunits to re-form 70S ribosomes. The results from sucrose gradient analysis indicated that 27–50% reassociation

Table I: Protein Synthesis Activity of Reconstituted Particles[a]

treatment	[^{14}C]poly-(phenylalanine) (cpm/A_{260})	% act.
−trioxsalen −light	10234	100
+trioxsalen −light	10770	105
−trioxsalen +light	11178	109
+trioxsalen +light	869	8
+trioxsalen +light[b]	9184	90

[a] Poly(uridylic acid)-directed [^{14}C]phenylalanine incorporation into poly(phenylalanine) with reconstituted particles and native 50S subunits was performed as described under Materials and Methods. The 16S RNA was treated with trioxsalen, light (22 × 10^4 J/m^2), or both as indicated, under identical conditions. The incorporation is given as counts per minute of [^{14}C]phenylalanine per A_{260} unit of 16S RNA, determined by formamide gel analysis (see Materials and Methods). [b] In this case, 30S subunits were treated, and the 16S RNA was isolated and used for the reconstitution and activity assays as described.

Table II: Protein Composition of Reconstituted Particles[a]

protein	30 S	22 S	22 S/30 S	protein	30 S	22 S	22 S/30 S
S1	0.9	0.7	0.80	S11 + S12	11.0	11.8	1.07
S2	2.4	1.6	0.66	S13	3.4	4.6	1.32
S3	6.7	4.5	0.67	S14	3.4	2.1	0.60
S4	11.8	13.5	1.15	S15	7.0	13.8	1.99
S5	5.6	9.8	0.86	S16	4.5	3.4	0.76
S6	4.0	4.1	1.00	S17	1.7	1.7	1.00
S7	12.1	11.4	0.94	S18	2.8	3.3	1.16
S8	2.9	3.5	1.23	S19	4.6	4.1	0.90
S9	4.9	5.7	1.16	S20	1.3	1.8	1.36
S10	1.2	0.6	0.50	S21	1.1	1.8	1.65

[a] 30 S and 22 S refer to particles reconstituted with ^{14}C-labeled proteins and native or trioxsalen-treated 16S rRNA as described under Materials and Methods. The relative amount of each protein is given as the percent of the total ^{14}C radioactivity as determined by summing the values for each stained spot in the gel. For the two gels the totals were 13700 cpm (30 S) and 10012 cpm (22 S). For each protein the amount in the 22S particle relative to that found in the 30S particle is given by the ratio in the last column.

occurred (depending upon the extent of purification of the particles). The 22S particles formed with trioxsalen-treated 16S RNA showed no reassociating capacity under the same conditions (data not shown).

Effects of Trioxsalen Treatment on 16S RNA within 30S Subunits. Ion-depleted and ion-activated 30S subunits contain 16S RNA in "open" and "closed" forms, respectively (Hochkeppel & Craven, 1976). Under either condition 16S rRNA in 30S particles was considerably less affected by trioxsalen than comparably treated protein-free RNA. [^{3}H]Trioxsalen binding to the RNA in 30S subunits treated at the saturation dose (12.5 × 10^5 J/m^2) was insignificant (<0.1 molecule/molecule of RNA). On sucrose gradients no radioactivity cosedimented with the RNA extracted from treated open or closed 30S ribosomes. At the same dose of light (22 × 10^4 J/m^2) used in the previous experiments, RNA treated within the 30S particle showed no alteration of either its ultraviolet absorption or fluorescence spectrum. The T_1 ribonuclease digestion kinetics of RNA isolated from treated particles were identical with those of native and control 16S RNA samples. In addition, particles sedimenting at 30S were

reconstituted from this RNA, and they had 90% of the activity of 30S particles reconstituted from control 16S RNA (Table I).

Discussion

These studies have shown that the binding of a relatively few molecules of trioxsalen to 16S rRNA can produce significant structural and functional alterations in the macromolecule. Extensive drug treatment of the RNA produced a small blue shift in the ultraviolet absorption maximum and a noticeable increase in the 235-nm absorbance. Similar effects have been observed in the case of 8-methoxypsoralen-treated tRNA (Ou & Song, 1978), but the precise effects of bound psoralen on the optical properties of the RNA remain to be determined The dose-dependent fluorescence at 395 nm after trioxsalen and light treatment of 16S RNA indicates the formation of monoadducts at the 4′,5′ position (Musajo & Rodighiero, 1972). The rate of increase of this fluorescence was about two times faster than the rate of increase of binding of the radioactive drug. Comparable differences in the rates of fluorescence evolution and drug binding with increased light doses have also been observed in the case of DNA (Musajo et al., 1966). A decline in fluorescence after prolonged treatment was observed by these authors. This point has not been examined in this study.

Alterations in environmental condtions which affect the secondary structure of RNA can be detected as changes in the ultraviolet absorbance of the molecule (Cox & Littauer, 1962; Boedtker, 1967; Araco et al., 1975). Thermal- or formaldehyde-induced denaturation of trioxsalen-treated 16S RNA promoted a reduction in hyperchromicity and an increase in T_m compared to untreated RNA. These differences have been interpreted as an indication of increased stabilization of the secondary structure of the drug-treated RNA, due to trioxsalen cross-link formation in double-stranded regions of the macromolecule (Karathanasis, 1978). Similar stabilization of DNA duplexes by trioxsalen treatment has been reported (Cole, 1970).

A marked resistance to digestion by ribonuclease T_1 was a distinct characteristic of trioxsalen-treated RNA, as indicated by the increased half-life for its degradation. A comparison of the kinetics of acquisition of RNase resistance and of isotopic drug binding indicated that the presence of a relatively few covalently bound trioxsalen molecules could produce a significant resistance to T_1 digestion. T_1 ribonuclease will only degrade single-stranded regions of RNA, since it has been reported that this enzyme is inactive on double-stranded RNA (Billeter et al., 1966). It has also been shown that under restrictive digestion conditions nucleolytic cuts are preferentially introduced into certain sensitive portions of 16S RNA (Muto et al., 1974). On the basis of these findings and the proposed loop–stem secondary structure of 16S RNA (Ehresmann et al., 1975), the observed resistance could be explained as follows.

If we assume that the most sensitive portions of the molecule are the single-stranded regions which connect the different loop-stem structures (interloop regions), then trioxsalen cross-links within the stem regions should not affect the T_1 digestion kinetics. Enzymatic cuts in the interloop regions would lead to the production of independent fragments and to a rapid reduction in the amount of intact 16S RNA as analyzed in the denaturing gels. In this case the $t_{1/2}$ for T_1 degradation should be identical for trioxsalen-treated and control RNAs.

If, however, we assume that the most sensitive portions of the RNA are located in single-stranded regions within the loops (intraloop single strands), then cross-links in the respective stems would prevent fragment dissociation under denaturing conditions. Ribonuclease-cleaved molecules would thus appear to migrate as intact 16S RNA in the gel. In this case an increase in the $t_{1/2}$ for drug-treated RNAs would be expected. This latter assumption is most consistent with our experimental observations.

We therefore interpret our data as additional evidence for the presence of two distinct T_1 sensitive sites in 16S RNA with the more sensitive sites located in the intraloop single-stranded areas of the molecule. Based on this interpretation, the "hidden breaks" described by Muto et al. (1974) should be mainly intraloop nucleolytic cleavages and the independent fragments would be generated from interloop scissions.

The fact that a small number of bound trioxsalen molecules can provide a substantial resistance to the RNA against T_1 degradation could suggest that the cross-links have occurred in the stems of the loops containing the most sensitive single-stranded segments. It is also possible that cross-linking of noncontiguous sequences of the RNA has occurred. Tertiary interactions leading to hydrogen bonding of noncontiguous sequences have been shown to occur in tRNA (Rich & RajBhandary, 1976) and have been postulated for rRNA (Ehresmann et al., 1975; Brimacombe et al., 1978). An examination of the sequences of the T_1 fragments of trioxsalen-treated RNA should help to substantiate these possibilities.

Trioxsalen treatment also influenced several functional characteristics of the 16S RNA. Reconstitution of trioxsalen-reacted RNA gave slowly sedimenting particles containing all of the 30S proteins. Several of the proteins which add to the 30S particle late in the assembly sequence (Held et al., 1974) were present in reduced amounts in the 22S particles. It appears that the presence of a limited number of trioxsalen adducts on the 16S RNA does not significantly affect its protein binding capacity. Furthermore, the reduced sedimentation coefficient indicates that the 22S particles must contain RNA in a more expanded or unfolded form compared to that present in intact or reconstituted 30S subunits (Miall & Walker, 1969; Eilam & Elson, 1971). The accumulation of 22S particles may indicate that a critical structural transition in the assembly sequence has been halted because of inflexible, trioxsalen-modified regions of the 16S RNA. These observations are consistent with the idea that during assembly, the rRNA is under continuous protein-mediated conformational alterations (Kurland, 1977; Brimacombe et al., 1978). The significance of conformational alterations in 16S RNA during 30S subunit assembly is emphasized by the large change in the sedimentation coefficient (21S to 26S) which accompanies the RI to RI* transition (Held & Nomura, 1973). These two intermediate particles of the in vitro assembly sequence have the same protein composition.

The observed instability of the 22S particles may reflect a competition between the binding of ribosomal proteins (and their eventual protective effect on the 16S RNA) and the hydrolysis of the nonprotected RNA by a contaminating ribonuclease in the 30S protein preparation. This is consistent with the observation that no breakdown of treated RNA occurred when incubated alone at 40 °C or with proteins at 0 °C. The RNA instability may be a consequence of the abnormal RNA structure in the particle. A preliminary examination of the properties of this nucleolytic activity excludes all the known *E. coli* ribonucleases except IV and N (Misra et al., 1976).

Another indication of the loss of function of the drug-treated 16S RNA was the inactivity of the 22S particles in protein synthesis. Less than 10% of the control activity was found when these particles were used in this activity assay. 22S particles were also defective in their ability to reassociate with native 50S subunits to promote the formation of 70S ribosomes. Thus, both structural and functional characteristics of 16S RNA were altered by trioxsalen treatment.

The fact that only a few molecules of bound trioxsalen could inactivate the capacity of 16S RNA to form 30S particles during reconstitution is consistent with other observations on modified RNA. Reconstitution has also been shown to be affected by the modification of 6–8 nucleotides in the 16S RNA by nitrous acid (Nomura et al., 1968). As in the present case, this treatment also produced more slowly sedimenting particles, with a complete protein complement, which were devoid of activity in protein synthesis assays.

In contrast, these effects on the 16S RNA were not observed when 30S subunits were treated with trioxsalen in either ion-depleted or ion-activated forms. In structural and functional tests 16S RNA "within" the subunit was considerably less affected by treatment with trioxsalen. Differences in drug binding have also been observed in the case of free and chromatin-bound DNA (Hyde & Hearst, 1978). These authors suggested that the low photoreactivity of DNA within the chromatin structure might be due to the greater stability of the molecule in the presence of histone proteins.

There are two general explanations for this difference in trioxsalen reactivity. Proteins bound on the nucleic acid may prevent the free exchange of drug between the nucleic acid and the solution (masking effect). Consequently, the local concentration of trioxsalen within the complex is reduced, and thus the extent of trioxsalen binding under exposure to light is decreased. Alternatively, the trioxsalen exchange between solution and nucleic acid is not altered, but due to the particular protein–nucleic acid interaction, the extent of secondary structure of the nucleic acid is substantially changed (structural effect). Consequently, less trioxsalen can be intercalated, causing a reduction in the extent of drug photobinding after exposure to light.

If our observation of substantially different trioxsalen photobinding represents differences in the secondary or tertiary structure of the RNA (depending upon the presence or absence of proteins), as others have suggested (Miall & Walker, 1969; Bollen et al., 1970; Araco et al., 1975), then the trioxsalen photoreaction may be useful for defining the state of the rRNA under a number of different conditions. Variations in ionic strength and temperature (known to influence the RNA conformation; Cox & Littauer, 1962; Morris et al., 1975) can be examined with regard to trioxsalen binding and the configuration of the RNA. The state of the RNA after interaction with individual ribosomal proteins or as it exists in intermediate precursor particles of the ribosomal subunits can also be examined with this photochemical probe. It should also be possible to distinguish between the open and closed conformations of the RNA described by Hochkeppel & Craven (1976). Finally, if noncontiguous sequences of the RNA are hydrogen-bonded within the 30S subunit (tertiary interactions; Ehresmann et al., 1975; Brimacombe et al., 1978), it may be possible to identify these sequences by using more reactive trioxsalen derivatives.

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Conformation of *Escherichia coli* Ribosomal Protein L7/L12 in Solution: Hydrodynamic, Spectroscopic, and Conformation Prediction Studies†

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ABSTRACT: The conformation of *Escherichia coli* ribosomal protein L7/L12 in solution has been studied using spectroscopic and hydrodynamic methods. Circular dichroism studies in the near-ultraviolet region reveal two bands at 262 and 268 nm originating from the tertiary conformational environment of the phenylalanyl residues. Additional characterization of the phenylalanine environment includes an intrinsic fluorescence emission spectrum arising from the phenylalanine fluorophores. Computer analysis of the far-ultraviolet circular dichroism spectrum suggests that L7/L12 contains as much as ~76% α helix. Hydrodynamic properties of L7/L12, measured with the purpose of providing relevant shape information, include the frictional coefficient ratio (1.84 ± 0.03) and intrinsic viscosity (28 ± 0.4 mL/g). The experimentally determined frictional coefficient ($6.15 \pm 0.15 \times 10^{-8}$) has been compared with theoretical calculations of the same value employing two independent methods and assuming various dimensions for the L7/L12 dimer. Combining the experimental results from this work with those available from the literature, and using conformation predictive methods of Chou & Fasman [P. Y. Chou & G. D. Fasman (1974) *Biochemistry 13*, 211–222, 222–245] and of Maxfield & Scheraga (F. R. Maxfield & H. A. Scheraga (1976) *Biochemistry 15*, 5138–5153), several possible molecular models of the L7/L12 dimer have been constructed and critically examined. A model which is consistent with all of the available data is proposed.

The elucidation of the molecular mechanism of ribosome self-assembly is a monumental task. The molecular components that make up the prokaryotic 70S ribosome from *Escherichia coli* include some 55 proteins and three species of RNA, providing the potential for a tremendous variety of inter- and intramolecular interactions. A prerequisite to the successful investigation of ribosomal self-assembly, however, is an understanding of the detailed molecular structures of the component proteins and RNAs.

Proteins L7 and L12, from the 50S subunit of *E. coli* ribosomes, were the first ribosomal proteins to be completely sequenced (Terhorst et al., 1972a, 1973). It was found that the two sequences were identical except for the presence of an acetylated amino-terminal serine group on L7 (Terhorst et al., 1972b). For this reason, they are commonly referred to as a single protein termed L7/L12. Considerable information has accumulated in the literature concerning the functional importance of this ribosomal protein, indicating that the presence of L7/L12 is required for the ribosome to carry out the initiation, elongation, and termination steps of protein synthesis (for a review, see Möller, 1974). Structural information about L7/L12, then, is necessary for a better understanding not only of the process of ribosomal self-assembly, but also of its important functional roles.

Early attempts to analyze the secondary structure using circular dichroism reveal that L7/L12 contains substantial amounts of α helix, estimates varying from 45 to 60%. These estimates, however, are calculated from single wavelength information and are based upon comparisons with poly-(α-amino acids) (Möller et al., 1970; Dzionara, 1970; Boublik et al., 1973). In addition, some of these measurements were obtained under denaturing conditions (Dzionara, 1970). Detailed analyses of circular dichroism data as well as other spectroscopic properties of protein L7/L12 obtained under functionally relevant conditions are not yet available. Pre-

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