Ribonucleic Acid–Protein Cross-Linking in *Escherichia coli* Ribosomes: (4-Azidophenyl)glyoxal, a Novel Heterobifunctional Reagent†

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ABSTRACT: We have used the heterobifunctional reagent (4-azidophenyl)glyoxal (APG) to cross-link RNA to protein in *Escherichia coli* 30S ribosomal subunits. Synthesis and characterization of the reagent are described. Like other dicarbonyl reagents (e.g., kethoxal), APG reacts specifically with guanosine among the four ribonucleosides. The azido group in APG can be photolyzed with UV light ($\lambda > 300$ nm), yielding an unstable nitrene which is potentially reactive with many groups in proteins and nucleic acids. Conditions for APG modification of guanylic acid residues in 30S subunits are described; photolysis of bound APG results in cross-linking of approximately 5% of the total 30S proteins to 16S RNA. A specific subset of the 30S proteins is cross-linked to 16S RNA by APG.

Ribosomes are the largest and most complex components of the cellular machinery for protein synthesis. For example, the *E. coli* ribosome is composed of 52 different protein molecules and three RNA molecules, organized into two structurally and functionally distinct subunits. Determining the three-dimensional structure of the ribosome is a prerequisite for complete understanding of the mechanism of protein synthesis, but the complexity and asymmetry of the structure present major barriers to its solution.

Nevertheless, major advances have been made by using newly developed approaches. There is now a satisfying level of agreement on the detailed locations of some of the ribosomal proteins in a three-dimensional model of the ribosome (Moore, 1980; Lake, 1980). In contrast, detailed information exists for only one site in ribosomal RNA, the 3′ terminus of 16S RNA, which has been localized to the same region of the *E. coli* 30S ribosomal subunit in four independent studies (Politz & Glitz, 1977; Olson & Glitz, 1979; Shatsky et al., 1979; Stöffler et al., 1980). Possible locations of other domains in ribosomal RNA have been proposed, but the evidence for these inferences has been of necessity indirect (Zimmerman, 1980; Bogdanov et al., 1980; Noller, 1980).

The need for direct examination of the structural disposition of ribosomal RNA in the ribosome has led to the development of new methods for determining the physical proximity of specific ribosomal proteins to specific sites in ribosomal RNA. Principal among these new approaches has been the exploitation of photochemical and chemical cross-linking of ribosomal proteins to ribosomal RNA [reviewed in Zimmermann (1980)]. Either by direct UV[1] irradiation of ribosomes or by use of bifunctional cross-linking reagents, several groups have succeeded in cross-linking proteins to ribosomal RNA. In only one case, however, has the subsequent analysis of cross-linked products been carried through to unambiguously identify the specific cross-linked peptide and oligonucleotide (Möller et al., 1978; Zwieb & Brimacombe, 1979).

Direct UV irradiation of ribosomes at low doses cross-links only three specific proteins to ribosomal RNA (Möller & Brimacombe, 1975; Baca & Bodley, 1976; Möller et al., 1978). Higher doses cause such extensive cross-linking that cooperative unfolding of subunits appears to occur (Möller & Brimacombe, 1975; Gorelic, 1976). Bifunctional chemical reagents for cross-linking have been less extensively exploited, largely due to the scarcity of suitable reagents (Zimmermann, 1980; Ulmer et al., 1978). We have developed a bifunctional reagent, (4-azidophenyl)glyoxal (APG), which has potential for investigation of RNA–protein topography in the ribosome. Like other dicarbonyl reagents (e.g., kethoxal), APG should react with guanosine (Staehelin, 1959; Shapiro & Hachmann, 1966; Litt & Hancock, 1967; Noller, 1974) at sites in RNA which are not involved in base-pairing interactions (Litt & Hancock, 1967; Litt, 1969; Noller, 1974). Thus, a relatively small number of sites in ribosomal RNA should be modified, minimizing effects of the reagent on ribosome conformation. Once APG is covalently bound to ribosomes via the dicarbonyl group, the azide functional group can be selectively photolyzed with long wavelength ultraviolet light, generating an unstable nitrene which is potentially reactive with many chemical groups in both proteins and nucleic acids (Bayley & Knowles, 1977). Since cross-linking is a two-step reaction, unbound APG can be removed before photolysis, thereby minimizing the cooperative unfolding and aggregation problems encountered with other reagents (Baumert et al., 1978; Ulmer et al., 1978). Furthermore, the extensive information obtained in this laboratory on sites of kethoxal modification in *E. coli* 16S and 23S ribosomal RNA should facilitate analysis of the cross-linked RNA (Noller, 1974; Chapman & Noller, 1977; Hogan & Noller, 1978; Herr & Noller, 1978).

We describe here the synthesis and characterization of APG, its reaction with guanosine, RNA, and ribosomes, and its properties in cross-linking protein to RNA in the *E. coli* 30S subunit. We also report the preliminary characterization of several proteins cross-linked to 16S RNA by APG treatment of 30S subunits.

Experimental Section

Analytical Methods. Ultraviolet spectra were taken on a Cary 14 spectrophotometer and infrared spectra on a Perkin-Elmer 237B instrument. Nuclear magnetic resonance

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[1] Abbreviations used: APG, (4-azidophenyl)glyoxal; UV, ultraviolet; butyl-BPD, 2-(4-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-dioxazole; Me_2SO, dimethyl sulfoxide; TLC, thin-layer chromatography; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid, disodium salt; NMR, nuclear magnetic resonance; PPO, 2,5-diphenyloxazole.

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(NMR) spectra were obtained on a JEOL JNM-FX60 instrument. Microanalyses were performed on a Perkin-Elmer Model 240B elemental analyzer. ^{32}P radioactivity in nonaqueous samples was measured in 10 mL of a cocktail containing 8.4 g of butyl-BPD (Beckman) per liter of toluene. ^{35}S radioactivity in aqueous samples was measured in 10 mL of the same cocktail plus 10% (v/v) Biosolv (Beckman). A Beckman LS-200B liquid scintillation system was used.

Synthesis of (4-Azidophenyl)glyoxal. A 1.5-g portion of selenium dioxide (Matheson, Coleman and Bell) was dissolved in 7.3 mL of dioxane plus 0.25 mL of water by heating to 80 °C. A 2.0-g portion of 4-azidoacetophenone (Pochinok & Kalashnikova, 1954; Hepher & Wagner, 1960) was dissolved in 1 mL of dioxane and then added dropwise to the SeO_2 solution. The reaction mixture was refluxed (100–110 °C) with stirring for 3.5 h and then filtered (Whatman no. 1) to remove solid selenium. (Note: Selenium and its compounds are toxic. Contact with skin was avoided; solid and liquid wastes contaminated with selenium were disposed of by institutional Environmental Health and Safety personnel.) After concentration of the filtrate in vacuo, the resulting oil was heated in 5 volumes of boiling water until no more would dissolve. A residual immiscible oil was removed from the bottom of the flask. The product, (4-azidophenyl)glyoxal monohydrate, crystallized from the aqueous solution after standing 48 h at 20 °C (600 mg, 25%). Recrystallization from benzene gave a melting point of 96–98 °C. The ^1H NMR data were the following: (Me_2SO-d_6) δ 5.63 [br t, 1, $CH(OH)_2$], 6.74 [d, 2, $CH(OH)_2$], 7.23 (d, 2, aromatic), 8.13 (d, 2, aromatic); (Me_2SO-d_6 plus 1 drop of D_2O) δ 5.63 [s, 1, $CH(OD)_2$], 7.23 (d, 2, aromatic), 8.13 (d, 2, aromatic). Other spectral data were the following: IR 2131 (N_3), 1695 (CO) cm^{-1}; UV (0.2% ethanol in H_2O) λ_{max} 296 nm (ϵ_{max} 2.33 × 10^4). Thin-layer chromatography of (4-azidophenyl)glyoxal hydrate [silica gel G, diethyl ether–hexanes (1:1)] gave an R_f of 0.40, compared to 0.88 for 4-azidoacetophenone. Anal. Calcd for $C_8O_2N_3H_5 \cdot H_2O$: C, 49.74; H, 3.63; N, 21.76. Found: C, 49.06; H, 3.59; N, 21.97.

Reaction of APG with Nucleosides and Nucleotides. Guanosine was reacted for 2 h at 37 °C with kethoxal or APG in 100 μL of solution containing 45% aqueous methanol, 0.1 μmol of guanosine, and 0.9 μmol of APG or 2.6 μmol of kethoxal. Aliquots of 25 μL of reaction mixtures were chromatographed on cellulose thin-layer sheets with 2-propanol–water (7:3) as eluant. Guanosine and reaction products were detected by viewing the chromatogram under ultraviolet light.

Mononucleotides obtained from alkaline hydrolysis of ^{32}P-labeled *E. coli* 16S ribosomal RNA (specific activity approximately 10 000 cpm/μg) were separated by paper electrophoresis, eluted, and concentrated by lyophilization (Barrell, 1971) before reaction with APG. Reaction mixtures contained 1500 cpm of ^{32}P-labeled UMP, GMP, AMP, or CMP and 0.1 μmol of APG (0 μmol of APG in controls) in 15 μL of buffer F. Reaction mixtures were incubated for 1 h at 37 °C and then electrophoresed at pH 3.5 for 1.5 h at 3000 V (Barrell, 1971) on Whatman 3MM paper. Following autoradiography, spots were cut out from the paper and counted.

Solution Properties of APG. APG was routinely kept at −20 °C as a 200 mg/mL dioxane solution. Before use for chemical modification, the stock dioxane solution was diluted to an APG concentration of 6 mg/mL in buffer E. APG immediately precipitated but was redissolved by heating 5 min at 65 °C. After brief centrifugation to remove remaining traces of precipitate, the appropriate volume of APG solution was added to each sample containing ribosomal subunits. APG did not reprecipitate after cooling to room temperature for at least 30 min.

Buffers. Buffer A: 0.1 M NH_4Cl, 0.01 M $MgCl_2$, 0.01 M Tris (pH 7.5), 1 mM EDTA, and 6 mM 2-mercaptoethanol. Buffer B: 0.5 M NH_4Cl, 0.01 M $MgCl_2$, 0.02 M Tris (pH 7.5), 0.5 mM EDTA, and 6 mM 2-mercaptoethanol. Buffer C: 0.03 M NH_4Cl, 1 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 6 mM 2-mercaptoethanol. Buffer D: 0.03 M NH_4Cl, 10 mM $MgCl_2$, 0.02 M Tris (pH 7.5), and 6 mM 2-mercaptoethanol. Buffer E: 0.1 M sodium cacodylate (pH 7.0), 0.02 M sodium borate, and 0.01 M $MgCl_2$. Buffer F: 0.1 M sodium cacodylate (pH 7.0), 0.02 M sodium borate, and 1 mM EDTA. Buffer G: 0.1 M sodium acetate (pH 6.0), 0.02 M sodium borate, 0.1 M LiCl, 1 mM EDTA, and 0.5% $NaDodSO_4$. Buffer H: 0.1 M sodium acetate (pH 6.0), 0.02 M sodium borate, 0.1 M LiCl, 1 mM EDTA, and 0.1% $NaDodSO_4$.

Enzymes. Ribonuclease T1 (Sankyo) was obtained from Calbiochem, ribonuclease A from Worthington, bacterial alkaline phosphatase from Sigma, and ribonuclease T2 (Sankyo) from Calbiochem. Standard enzyme solutions were prepared according to Barrell (1971).

Preparation of Ribosomal Subunits and Proteins. For ^{32}P-labeled ribosomes, *E. coli* MRE600 cells were grown on low-phosphate medium (Garen & Levinthal, 1960) as described (Chapman & Noller, 1977). A 50-mL culture was supplemented with 0.5–10 mCi of carrier-free [^{32}P]orthophosphate. Cells were harvested, washed with buffer A, and lysed by rapid freezing and thawing of the pellet, followed by grinding with an equal weight of alumina for 5 min. Ribosomal subunits were isolated according to Noller (1974), except that the sucrose gradient buffer contained 1 mM $MgCl_2$ (buffer C).

For ^{35}S-labeled ribosomes, strain MRE600 cells were grown on a low-sulfate medium as described (Sun et al., 1974). A 50-mL culture was supplemented with 10–20 mCi of carrier-free $H_2{}^{35}SO_4$. After the cells were harvested, they were lysed by the lysozyme freeze–thaw method of Ron et al. (1966) in the presence of buffer A. Ribosomal subunits were isolated as described above for ^{32}P-labeled subunits.

Unlabeled 30S ribosomal proteins were isolated from *E. coli* Q13 30S subunits (gift of Dr. Ferdinand Dohme) by extraction with acetic acid (Kurland et al., 1971) and stored at −20 °C in 0.01 M Tris-HCl, pH 7.5, and 6 mM 2-mercaptoethanol.

Modification of 30S Ribosomal Subunits with APG. An aliquot containing 20 μg of concentrated 30S subunits was diluted to 500 μL with buffer E. In analytical experiments with ^{35}S-labeled subunits, 7 × 10^4 cpm (less than 1 μg) of labeled subunits were mixed with 20 μg of unlabeled carrier subunits. In preparative experiments, 20 μg of ^{35}S-labeled subunits was used alone. A 100-μL aliquot of 6 mg/mL APG in buffer E was added (in minus APG controls, 100 μL of buffer E was added). Reaction mixtures were incubated at 37 °C for 45 min and then put on ice. For removal of unbound APG, subunits were precipitated twice with 0.65 volume of ethanol (−20 °C). Precipitates were resuspended in 600 μL of buffer E for photolysis of bound APG.

Photolysis of APG Bound to 30S Subunits. ^{35}S-Labeled subunits previously modified by APG were irradiated for up to 120 min in 1.5-mL polypropylene microcentrifuge tubes with tube caps open (Rabin & Crothers, 1979). Samples were irradiated from above in a room-temperature water bath by a 9 W, 12-in. "long-wavelength" UV tube (Edmund Scientific No. 60 889), placed 2 mm above the row of sample tubes. A Pyrex petri dish could be placed between source and sample to cut off light below 300 nm.

Detection and Quantitation of Proteins Cross-Linked to 16S RNA by APG. After photolysis, subunits were again precipitated with ethanol and resuspended in 50 μL of buffer G. To measure the amount of protein bound to 16S RNA, each sample was layered on a separate 3.4-mL 5–20% sucrose gradient in buffer H. Gradients were centrifuged at 55 000 rpm for 2.5 h at 10 °C (IEC B-60 ultracentrifuge, SB-405 rotor). Gradients were fractionated, and ^{35}S radioactivity was measured in each fraction.

Characterization of Cross-Linked Proteins. A 20-μg sample of ^{35}S-labeled 30S subunits (20×10^6 cpm) was reacted with APG as described above. After photolysis, non-cross-linked proteins were removed in $NaDodSO_4$–LiCl sucrose gradients as described above, or in larger versions (35 mL, 10–30% sucrose in buffer H, 25 000 rpm for 24 h at 10 °C, SW 27 rotor). Fractions containing the center of the 16S peak were pooled. Unlabeled 30S subunits (20 μg) were added, and the 16S RNA was precipitated with 2 volumes of ethanol after the Mg^{2+} concentration was raised to 10 mM. After several hours at −20 °C, the RNA was pelleted (Sorvall HB-4 rotor, 8500 rpm for 4.5 h at 4 °C). The RNA pellet was redissolved in 20 μL of water, and 100 μL of the RNA solution was digested for 3 h at 37 °C with 10 μL of a solution containing 0.1 mg/mL RNase T1 and 1.0 mg/mL bacterial alkaline phosphatase and 10 μL of a 0.1 mg/mL solution of RNase A. Ribosomal proteins in the digested sample were precipitated at −20 °C with 5 volumes of acetone in the presence of 280 μg of added unlabeled total 30S protein (*E. coli* Q13). The pellet was centrifuged out and redissolved in 50 μL of 0.01 M Tris-HCl, pH 7.5, 6 mM 2-mercaptoethanol, and 8 M urea.

Proteins were separated by using the two-dimensional polyacrylamide–urea electrophoresis system of Kaltschmidt & Wittmann (1970). After electrophoresis, the gel was fixed in 5% trichloroacetic acid for 1 h and stained in 0.25% Coomassie Blue in 9.2% acetic acid and 45% methanol. After the gel was destained, it was impregnated with PPO according to Bonner & Laskey (1974) or was soaked in 90 mL of Enhance (New England Nuclear) for 2.5 h at 20 °C and then in water at 20 °C for 1 h. The gel was then dried overnight and fluorographed on Kodak XR-5 film for 4–45 days at −80 °C.

Detection of Cross-Linked Oligonucleotide–Protein Complexes. A 20-μg sample of ^{32}P-labeled 30S subunits (20×10^6 cpm) was cross-linked with APG as described above. After resuspension in 50 μL of buffer G, RNA in the sample was precipitated with 2.5 volumes of ethanol in the presence of 20 μg of added carrier 30S subunits (MRE600). The RNA pellet was digested for 1 h at 37 °C with 25 μL of a solution containing 1.2 μg of RNase T1, 12 μg of bacterial alkaline phosphatase, and 1.2 μg of RNase A. As above, ribosomal proteins and cross-linked nucleotides were concentrated by acetone precipitation in the presence of carrier MRE600 total 30S proteins, and the mixture was separated by two-dimensional electrophoresis. The destained gel was dried overnight and autoradiographed on Kodak XR-5 film for 7 days at −80 °C.

Quantitation of Modified Guanosine in APG-Modified 30S Subunits. Subunits labeled with ^{32}P were modified with APG as described for ^{35}S-labeled subunits. Each sample contained 20 μg of subunits (60×10^6 cpm). After the samples were reacted with APG (without UV photolysis), subunits were ethanol precipitated, resuspended in buffer H, and deproteinized in $NaDodSO_4$–LiCl sucrose gradients as described for ^{35}S-labeled subunits. The 16S peak fractions were pooled, and then RNA was recovered by ethanol precipitation and digested completely with RNase T2 (Barrell, 1971). The digestion products were separated by electrophoresis at pH 1.7 (7% formic acid, Whatman 3MM paper) for 45 min at 2000 V. After autoradiography, the unmodified mononucleotide and APG-modified spots were cut out and counted.

Table I: Thin-Layer Chromatography Results[a]

compound	R_f
guanosine	0.50
kethoxal–guanosine product	0.84
APG–guanosine adduct	0.88

[a] Reactions and chromatography are as described under Experimental Section.

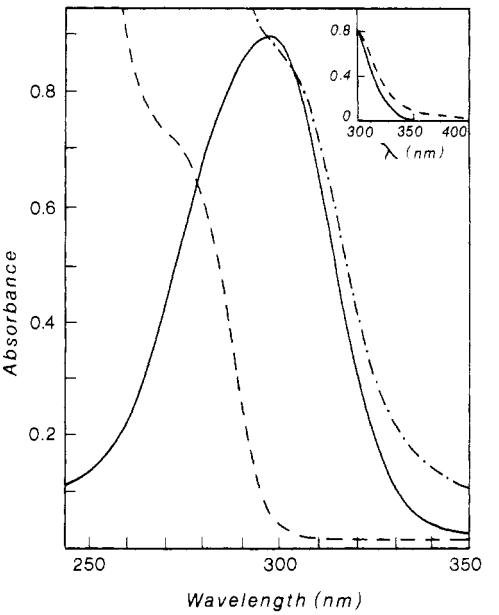

FIGURE 1: Ultraviolet spectra of guanosine (---), APG (—), and equimolar guanosine plus APG (-·-·-). All samples were incubated at room temperature for 24 h in buffer E at final equimolar concentrations of 1.67×10^{-4} M guanosine and APG. Samples were diluted to 3.3×10^{-5} M with buffer E just before recording spectra. Inset: Visible spectra of a 24-h reaction mixture of equimolar APG and guanosine (---) vs. an equimolar mixture of APG and guanosine just after mixing (—). Both samples were 3.3×10^{-5} M when spectra were recorded.

Results

The reagent (4-azidophenyl)glyoxal was obtained as the crystalline monohydrate in a three-step synthesis. APG was stable when stored as the solid at −20 °C for at least 18 months, as judged by thin-layer chromatography.

Reaction of APG with ribonucleosides and ribonucleoside monophosphates was examined by thin-layer chromatography, paper electrophoresis, and UV spectroscopy. Initially, reaction between guanosine and APG was compared to the reaction between guanosine and kethoxal, a well-characterized reagent for guanosine modification in nucleic acids. Reaction of either APG or kethoxal with guanosine showed partial conversion of guanosine to a less polar product (Table I). The APG–guanosine adduct showed up as an intense green-yellow fluorescent spot when viewed under UV light.

Ultraviolet and visible spectra of APG–guanosine reaction mixtures were compared with spectra of APG and guanosine (Figure 1). After 24 h at room temperature, the reaction mixture showed a pronounced shoulder or tail (approximately 10% of the absorbance at 300 nm) compared with a spectrum of APG at the same molar concentration. This shoulder extended the reaction mixture spectrum out to about 400 nm

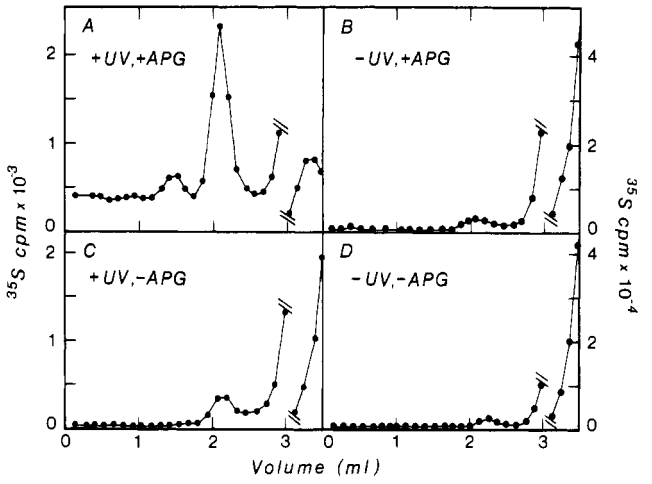

FIGURE 2: $NaDodSO_4$–LiCl sucrose gradient analysis of RNA–protein cross-linking in 30S subunits by APG. Details of chemical modification and sedimentation are described under Experimental Section. Each sample contained 7×10^4 cpm of ^{35}S radioactivity. Sedimentation was from right to left. Samples were derived from subunits that were (A) APG modified and UV photolyzed; (B) APG modified only; (C) UV photolyzed only; (D) unmodified. Photolysis in (A) and (C) was for 120 min.

whereas the APG spectrum showed zero absorbance in the region 350–400 nm. The inset to Figure 1 demonstrates that the shoulder is due to a reaction product, since a spectrum of guanosine plus APG taken immediately after mixing lacks this feature.

The specificity of APG for guanine was demonstrated in reaction with purified ^{32}P-labeled nucleoside monophosphates. Reaction products were separated by paper electrophoresis, pH 3.5. A modified spot appeared in the GMP–APG reaction mixture, migrating as an elongated spot between the positions of GMP and AMP. After reaction for 1 h at 37 °C with an excess of APG, 10.4% of the total radioactivity was converted to the modified spot. No modified spots were detected by autoradiography in similar reactions with UMP, AMP, and CMP.

Reaction of APG with guanylic acid residues in 30S subunits was measured. After dark reaction with APG, 16S RNA labeled with ^{32}P was extracted from 30S subunits and digested to mononucleotides with RNase T2. The mixture of mononucleotides was separated by paper electrophoresis at pH 1.7 (Bellemare et al., 1972). In this system, APG-modified guanylic acid has a mobility of 1.5 times that of guanylic acid. Compared to a control in which reagent was omitted, 0.26% of the total radioactivity appeared as modified guanylic acid, corresponding to an average value of 4 mol of guanosine modified per mol of 30S subunits.

When ^{35}S-labeled 30S subunits were modified with APG and then irradiated with ultraviolet light, proteins were cross-linked to 16S RNA. Cross-linking was measured as the amount of ^{35}S-labeled protein which comigrated with 16S RNA in $NaDodSO_4$–LiCl sucrose gradients (Figure 2). Such gradients rapidly and cleanly separate noncovalently bound ribosomal protein from 16S RNA. For example, a background of only 0.34% of total input radioactivity comigrated with the 16S peak when unmodified, unirradiated subunits were sedimented in this fashion (Figure 2D). In contrast, when subunits previously modified with APG were irradiated with UV light, 8.7% of the ^{35}S radioactivity comigrated with the 16S peak (Figure 2A). A small peak of radioactivity sedimenting faster than 16S and a higher background at the bottom of the gradient were usually observed with cross-linked subunits (e.g., Figure 2A). These effects are probably the result of cross-

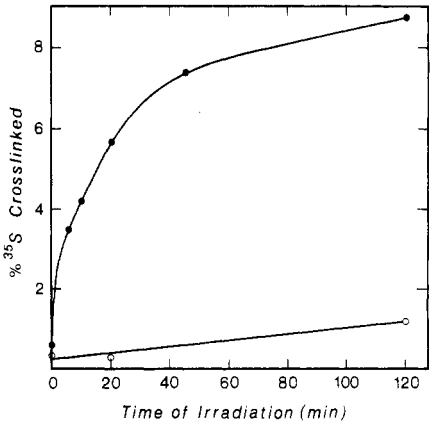

FIGURE 3: Time course of UV-dependent RNA–protein cross-linking. Percent cross-linking was measured in the standard $NaDodSO_4$–LiCl sucrose gradient assay. See Experimental Section for details. Each sample contained 7×10^4 cpm of ^{35}S radioactivity. (●) APG-modified subunits; (○) unmodified subunits.

linking the 30S–30S dimers or larger aggregates present at the 10 mM Mg^{3+} concentration in the reaction mixture. The appearance of higher aggregates at the expense of the 16S peak strongly depended on the reagent concentration; in order to optimize the yield in the 16S peak with respect to the larger aggregates, it was necessary to set the APG concentration for the modification reaction maximally at 1 mg/mL (data not shown).

Cross-linking was dependent on both treatment with APG and irradiation. Reaction with APG without subsequent irradiation caused only 0.60% of the ^{35}S radioactivity to comigrate with the 16S peak (Figure 2B) while irradiation in the absence of APG modification caused 1.14% comigration with the 16S peak (Figure 2C). This small amount of UV-dependent cross-linking could be reduced to background by irradiation for 20 min instead of the 120 min of Figure 2 (see Figure 3 and the following).

The kinetics of cross-linking were followed by using the sucrose gradient assay. Identical samples were modified with APG and then photolyzed for different lengths of time. Each sample was sedimented on a standard $NaDodSO_4$–LiCl sucrose gradient, and ^{35}S radioactivity in the 16S peak was measured. The time course of the cross-linking reaction is biphasic (Figure 3). The first, rapid phase is finished by approximately 30 min, after which cross-linking occurs only at a very slow rate, similar to that seen when unmodified subunits are irradiated (Figure 3, lower curve). Insertion of a Pyrex filter between UV source and sample to absorb light less than 300 nm in wavelength caused no additional reduction of background after 20 min of irradiation (data not shown). Because of the small amount of UV-dependent cross-linking observed after 120 min of photolysis (Figure 2C and Figure 3, lower curve), in subsequent preparative experiments, APG-modified subunits were photolyzed for only 20 min.

The ^{35}S-labeled proteins which comigrate with 16S RNA after APG modification and photolysis were isolated by using a preparative version of the $NaDodSO_4$–LiCl sucrose gradient. Fractions containing ^{35}S radioactivity in the 16S peak were pooled and concentrated; the material was then subjected to total RNase digestion and analysis by two-dimensional gel electrophoresis in the presence of stainable amounts of total 30S protein. Results of a typical experiment are shown in the fluorogram of Figure 4. Five radioactive spots correspond closely in position to the stained spots for S2, S3, S4, S5, and S12. Each of these radioactive spots overlaps with its corresponding stained protein and streaks toward the first-dimension

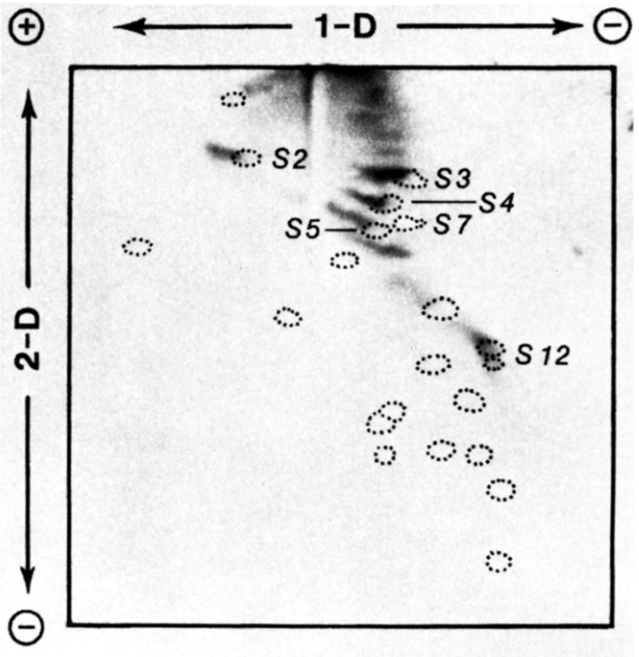

FIGURE 4: Fluorogram of a two-dimensional polyacrylamide electrophoresis gel of the ^{35}S-labeled 30S proteins which are cross-linked to 16S RNA by APG. Details are described under Experimental Section. The diagram shows the positions of unlabeled stained carrier 30S proteins from strain Q13 (dashed spots). Cross-linked proteins are identified by number.

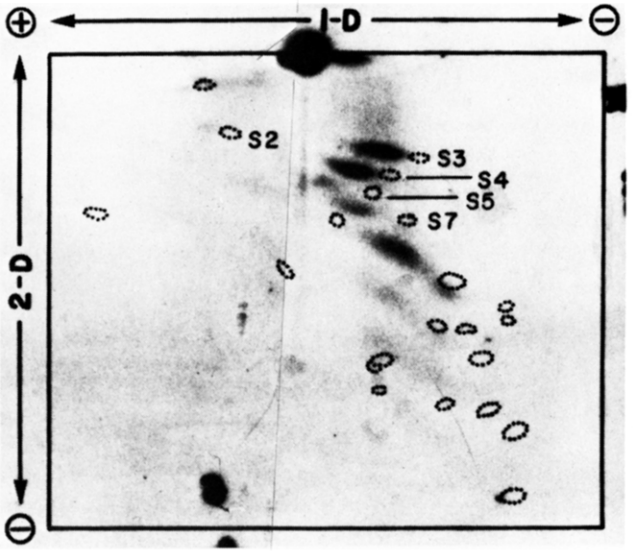

FIGURE 5: Autoradiogram of two-dimensional electrophoresis of ^{32}P-labeled nucleotides bound to 30S ribosomal proteins after APG cross-linking of ^{32}P-labeled subunits. Details are described under Experimental Section. The diagram shows the position of unlabeled stained 30S proteins from strain MRE600 (dashed spots). Cross-linked proteins are identified by number. The figure is a composite of two (left and right) autoradiograms of the same electrophoresis gel.

mode. The overall pattern of radioactive spots is clearly homologous to the corresponding pattern of stained spots. A sixth spot, most likely corresponding to protein S7, runs anomalously in a position below that of the stained spots for S5 and S7. This can be attributed to the well-documented difference in mobility of protein S7 derived from strain MRE600 (^{35}S-labeled proteins) and strain Q13 (stained proteins). The right-hand edge of the sixth spot corresponds closely to the position of protein S7 from strain MRE600 (Kaltschmidt et al., 1970; see also Figure 5).

In Figure 4, some ^{35}S-labeled cross-linked material migrates too slowly to be single 30S proteins. This material is probably not a large complex of both RNA and protein (see the same region of the gel in Figure 5). It could represent 30S proteins cross-linked to each other via APG-modified arginyl residues.

Four separate analyses of the cross-linked proteins were conducted. In one of the analyses, RNA was digested with RNase T2; in the other three, including the experiment of Figure 4, RNA was digested with a mixture of RNase T1, pancreatic RNase, and alkaline phosphatase. In each case, a similar pattern of fluorographed spots was observed (data not shown).

The experiment of Figure 4 was repeated, using ^{32}P-labeled subunits, to determine whether proteins from APG-cross-linked ribosomes contain covalently linked nucleotides which cannot be removed by RNase digestion. In the autoradiogram shown in Figure 5, a pattern of ^{32}P-labeled spots similar to that of the ^{35}S-labeled pattern of Figure 4 is found. In particular, ^{32}P spots homologous to the positions of stained proteins S2, S3, S4, S5, and S7 are evident. As in Figure 4, the labeled spots are elongated in the direction of the anode. In this case, the displacement of the ^{32}P spots from the stained pattern is exaggerated, possibly because the intensity of the label reflects the length of the attached RNA fragment as well as the relative amount of cross-linked complex. A few faster migrating ^{32}P-labeled spots can be seen which are diffuse and thus less easily related to the staining pattern.

Discussion

The specificity of reaction of APG with guanosine and guanosine monophosphate is similar to that of other dicarbonyl compounds (e.g., glyoxal, phenylglyoxal, and kethoxal). The guanosine–APG adduct is fluorescent, like the guanosine–phenylglyoxal adduct reported by Shapiro & Hachmann (1966).

We anticipated reaction of APG with 30S ribosomal subunits to occur between guanylic acid residues of 16S RNA and the dicarbonyl group of APG. That this takes place is demonstrated by the detection of a modified guanosine monophosphate in APG-modified, ^{32}P-labeled 30S subunits. Quantitatively, approximately four guanylic acid residues are modified per 30S subunit. It was important to estimate guanosine modification by APG because phenylglyoxal is a well-known chemical modification reagent for arginine residues in proteins (Takahashi, 1968). Although we have not attempted to measure APG modification of arginine in 30S proteins, our conditions have been chosen to minimize arginine modification. The rate constant for arginine modification by phenylglyoxal increases 40-fold with increasing pH in the range 7.5–11.5 (Takahashi, 1968, 1977; Cheung & Fonda, 1979) and decreases 12-fold in the presence of 50 mM borate ion (Cheung & Fonda, 1979). Conversely, the reaction of dicarbonyl compounds with guanosine is promoted by borate and neutral pH (Litt, 1969). Our conditions with respect to these variables are pH 7.0 and 0.02 M sodium borate, respectively.

Cross-linking of 30S proteins to 16S RNA occurs when APG-modified 30S subunits are irradiated with UV light. That this cross-linking involves photolysis of bound APG is suggested by the conditions required; i.e., both irradiation and prior APG modification are required for cross-linking. Irradiation alone produces no cross-linking, indicating that nucleic acids and proteins are probably not photolyzed by the irradiation conditions used. APG treatment without irradiation also produces no cross-linking, suggesting that no unexpected chemistry is involved.

The cross-linking yield (ca. 5%) represents roughly one protein molecule cross-linked per 16S RNA molecule or 30S

subunit, assuming an approximately homogeneous distribution of ^{35}S radioactivity in the 21 30S proteins. It is probably not desirable to promote more extensive cross-linking since this could result in disruption of the native ribosome structure.

After isolation of ^{35}S-labeled cross-linked proteins and digestion of the cross-linked RNA, most of the ^{35}S radioactivity migrates in discrete protein spots on two-dimensional electrophoresis gels, corresponding to a specific subset of the 30S ribosomal proteins. The ^{35}S-labeled spots are elongated in the direction of the anode, suggesting that the labeled proteins are covalently linked to residual tails of RNase-resistant oligonucleotides. This interpretation is confirmed by the experiment of Figure 5, which demonstrates that ^{32}P-labeled nucleotides are found in protein spots from APG-cross-linked 30S subunits on two-dimensional gels. Furthermore, the ^{32}P-labeled spots are shifted in the expected direction relative to the corresponding stained spots, i.e., toward the anode. Since the second-dimension electrophoresis (pH 4.5) is run toward the cathode, these ^{32}P-labeled spots cannot be free oligonucleotides.

The altered electrophoretic mobility of ribosomal proteins bearing negatively charged phosphates is well documented, in the field of both protein phosphorylation (Gressner & Wool, 1974) and ribosomal RNA–protein cross-linking (Möller & Brimacombe, 1975). The altered mobilities observed here in Figures 4 and 5 constitute strong evidence that RNA–protein cross-linking has occurred.

The ^{35}S-labeling results (Figure 4) suggest that proteins S2, S3, S4, S5, S7, and S12 are cross-linked to 16S RNA by APG. Cross-linking of S2, S3, S4, S5, and S7 is confirmed by the ^{32}P-labeling results of Figure 5. Of these proteins, S4 and S7 have been previously cross-linked to 16S RNA by other RNA–protein cross-linking techniques. It is not surprising that S4 is found as a protein cross-linked to 16S RNA, since it interacts strongly with 16S RNA [reviewed in Zimmermann (1980)] and may by itself have a great deal to do with stabilizing the shape of the 30S subunit (Garrett, 1979). S7 also binds to 16S RNA in the absence of other proteins (Nomura & Held, 1974), protects a large region of 16S RNA from RNase digestion (Muto et al., 1974), and has been cross-linked to U_{1239} of 16S RNA by UV irradiation (Möller et al., 1978). The other cross-linked proteins described here, S2, S3, S5, and S12, are poorly understood in terms of their interaction with RNA. Thus, APG cross-linking of these proteins to 16S RNA offers the opportunity to learn a great deal about ribosome structure. We are presently determining which sequences in 16S RNA are cross-linked to the various 30S proteins by APG. It may be possible to use this information in conjunction with ribosomal protein topography data to locate regions of the RNA chain in a three-dimensional model of the 30S subunit.

Besides S4 and S7, the other cross-linked proteins also deserve further mention because of their implication as components of ribosomal functional sites. Proteins S2 and S3 have been shown to be important for tRNA binding to the ribosome (Rummel & Noller, 1973; Thomas et al., 1975; Van Duin et al., 1972; Randall-Hazelbauer & Kurland, 1972). Protein S12 is altered in streptomycin-resistant and -dependent *E. coli* strains (Ozaki et al., 1969; Birge & Kurland, 1970); protein S5 is altered in spectinomycin-resistant mutants and in revertants from streptomycin dependence (Hasenbank et al., 1973; Bollen et al., 1969). Lake (1980) has suggested that S5 and S12 may be located at the tRNA recognition site of the 30S subunit. Of course, information about all the cross-linking sites will be important, but the identification of parts of 16S RNA which are near known ribosomal functional sites will be particularly interesting.

The potential of APG for structural analysis of nucleoproteins must, of course, be proven by application. These initial studies indicate that APG modification is promising as a general method for cross-linking nucleic acids to proteins in biological systems. It is applicable wherever guanosines in either DNA or RNA are in single-stranded conformations which are accessible to the dicarbonyl group. APG reacts under mild conditions of pH and temperature. The lack of specificity of the nitrene generated on photolysis is a valuable property, since one seldom knows the type of chemical groups which are present in the target environment. As a consequence, APG may also be useful as an RNA–RNA cross-linking reagent for studying such problems as the tertiary structure of the large ribosomal RNA molecules.

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Lipids of Synaptic Vesicles: Relevance to the Mechanism of Membrane Fusion†

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ABSTRACT: Synaptic vesicles from the electric organ of the marine ray *Narcine brasiliensis*, purified to at least 90% homogeneity, were analyzed for the lipid and fatty acid content of their membranes. The major lipids (mol %) were phosphatidylcholine (32.3%), phosphatidylethanolamine (20.5%), phosphatidylserine (6.1%), sphingomyelin (3.0%), and cholesterol (33.3%), a composition which did not differ greatly from that of the parent electric organ. While the number of double bonds per fatty acid molecule was similar for both synaptic vesicle and whole electric organ phospholipids, the vesicles were highly enriched in docosahexenoic acid (22:6). Reaction with the amine labeling reagents isethionylacetimidate and trinitrobenzenesulfonic acid indicated that 40% of the phosphatidylserine and 60% of the phosphatidylethanolamine are present on the external (cytoplasmic) surface of the synaptic vesicle. These data on a natural fusing membrane have relevance to models of membrane fusion, which have been based largely on studies of in vitro fusion using synthetic membranes.

In many types of secretory cells the secretory product is contained in membrane vesicles or granules and the release of the secretory product involves the fusion of the vesicle with the cell plasma membrane. The vesicle membrane constituents are at least transiently incorporated into the plasma membrane, and the contents are released into the extracellular space. In general this process of exocytosis is triggered by entry of calcium into the cell (Douglas, 1968; Llinas & Heuser, 1977; Holtzman, 1977). Exocytosis in vivo may involve proteins of the vesicle membrane and plasma membrane or the carbohydrate moieties of their glycoproteins and glycolipids. In vitro, however, model membrane studies [review, Papahadjopoulos et al. (1979)] have shown that membranes composed solely of purified lipids are capable of undergoing fusion. It has been

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