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Cross-Linking of Elongation Factor EF-G to the 50S Ribosomal Subunit of *Escherichia coli*†

A. Seetharama Acharya, P. B. Moore,* and F. M. Richards

ABSTRACT: A wide variety of bifunctional protein reagents will covalently cross-link the elongation factor EF-G to the 50S ribosomal subunit of *Escherichia coli*. About 120,000 daltons of ribosomal proteins become cross-linked to EF-G upon extensive reaction. About 30,000 daltons of this material is made up of proteins L7 and L12 (mol wt 13,000 daltons). Thus, these two components, whose presence on the ribosome is required for the EF-G interaction, must be located at the EF-G binding site.

There is reason to believe that elongation factors EF-G[1] and EF-Tu interact with the 50S subunit of the procaryotic ribosome at the same site or at very closely related sites. Antibiotics such as siomycin, thiostrepton, and thiopeptin which inhibit the interaction of EF-G with the ribosome also block the binding of aminoacyl-tRNA to ribosomes catalyzed by EF-Tu and its attendant hydrolysis of GTP (Bodley and Lin, 1970; Bodley *et al.*, 1970; Tanaka *et al.*, 1970; Weisblum and Demohn, 1970a,b; Pestka, 1970; Highland *et al.*, 1971; Kinoshita *et al.*, 1971; Modolell *et al.*, 1971a,b). Moreover, when EF-G is bound to the ribosome, EF-Tu will no longer interact with it (Richter, 1972; Miller, 1972; Cabrer *et al.*, 1972; Richman and Bodley, 1972). These results all seem to point to the existence of a critical region on the 50S subunit involved in both tRNA binding (the "A site") and translocation. Clearly it would be of greatest interest to know what the structure of the ribosome is in this region.

The one fact we do know about this binding center of the 50S subunit is that two 50S proteins, L7 and/or L12, must be present in order for its activities to be expressed. When these proteins are removed from the 50S subunit, the particle which remains is inactive with respect to both EF-G- and EF-Tu-catalyzed interactions (Hamel and Nakamoto, 1971, 1972; Hamel *et al.*, 1972; Sander *et al.*, 1972; Brot *et al.*, 1972). Proteins L7 and L12 (mol wt 13,000 each) have identical primary structures except that the N terminus of L7 is acetylated whereas that of L12 is free (Möller *et al.*, 1972; Terhorst *et al.*, 1972). The activity of particles lacking L7 and L12 can be restored by adding these components back. Recently Highland *et al.* (1973) have tested a large number of antibodies specific for 50S proteins for their ability to block the EF-G interaction. Only the antibodies raised against L7 or L12 have this effect. Accordingly, it has been suggested that L7 and L12 are in the EF-G binding site on the ribosome.

A direct method for finding out which ribosomal proteins are in the factor binding sites would be to identify the ribosomal components which can be cross-linked to factors with bifunctional reagents when they are bound to ribosomes. In this article we report the results of a study of the EF-G binding site using bifunctional reagents. The cross-linking of EF-G to the ribosome is demonstrated. Extensive cross-linking attaches about 120,000 daltons of 50S protein (which is about 20–25% of the total) to EF-G. Included in this material are about 2 equivalents of protein (L7–L12). This result proves that L7 and L12 are indeed at or very near the EF-G binding site.

Materials and Methods

Materials. Adiponitrile, suberonitrile, sebaconitrile, glutaraldehyde, and difluorodinitrobenzene were obtained from

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[1] Abbreviations used are: GMPPCP, β,γ-methyleneguanosine triphosphate; EF-G, peptide chain elongation factor G; EF-Tu, peptide chain elongation factor Tu.

Aldrich Chemical Co., Milwaukee, Wis. β,γ-Methyleneguanosine triphosphate (GMPPCP) was the product of Miles Laboratory, Kankakee, Ill. Bio-Gel A 1.5 m was the product of Bio-Rad Laboratories, Richmond, Calif. DEAE-Sephadex was purchased from Pharmacia, Uppsala, Sweden. [^{35}S]-Na_2SO_4 and tritiated amino acids were obtained from New England Nuclear, Boston, Mass. Dithiothreitol was a product of Calbiochem, Los Angeles, Calif.

Buffer systems used were: TM, 10 mM Tris–10 mM magnesium acetate, pH 7.5; buffer A, 10 mM Tris–10 mM magnesium acetate–10 mM ammonium acetate–1 mM dithiothreitol (pH 8.0); buffer B, 10 mM Tris–10 mM magnesium acetate–10 mM ammonium acetate–1 mM dithiothreitol (pH 8.0)–0.15 M KCl; buffer C, 10 mM Tris–10 mM magnesium acetate–10 mM ammonium acetate–1 mM dithiothreitol (pH 8.0)–0.3 M KCl; starting buffer, 6.0 M urea–3×10^{-2} M methylamine–6×10^{-3} M 2-mercaptoethanol, adjusted to pH 5.6 with acetic acid.

Cells and Ribosomes. Escherichia coli MRE 600 was grown and harvested as described previously (Moore, 1971). ^{35}S-Labeled MRE-600 was prepared by growing the cells on Hershey medium supplemented with [^{35}S]Na_2SO_4. Ribosomes were prepared by the method of Staehlin *et al.* (1969). Ribosomal subunits were isolated by sucrose gradient centrifugation (Moore, 1971). 50S ribosomal subunits were further purified by a second cycle of sucrose gradient centrifugation. The resulting subunit preparation showed no detectable 30S contamination upon sucrose gradient analysis (<3% by weight). Ribosomes as well as ribosomal subunits were stored under liquid nitrogen in buffer A.

Preparation of ^{35}S-Labeled EF-G. ^{35}S-Labeled factor G was prepared from ^{35}S-labeled MRE-600 cells. The procedure described by Gordon (1971) was used in the present study. Alumina ground cells were extracted with buffer A, and the protein and nucleic acids were partitioned using a poly(ethylene glycol)–dextran two-phase system. The proteins were recovered from the poly(ethylene glycol) phase by ammonium sulfate precipitation. The ammonium sulfate precipitated protein was back extracted with 27.8, 24, and 19.6% ammonium sulfate successively. EF-T is extracted by 24% ammonium sulfate whereas EF-G is extracted by 19.6% ammonium sulfate. The transfer factors were further purified by chromatography on DEAE-Sephadex at pH 8.0. EF-G was eluted from the column stepwise with 0.35 M NaCl in 10 mM Tris (pH 8.0)–1 mM dithiothreitol. Final purification was carried out on hydroxylapatite columns using the phosphate buffer system of Gordon (1971). The product that comes out of the hydroxylapatite column after the second chromatography has been reported to be free of contamination from EF-Tu (Gordon, 1971). Upon sodium dodecyl sulfate gel electrophoresis, it appeared to be 80–85% pure.

Preparation of 50S Ribosome–EF-G–GMPPCP Complex. 50S ribosomes and EF-G were dialyzed overnight against buffer A. In a typical experiment 15 $OD_{260m\mu}$ units of 50S ribosomes was incubated with a fourfold molar excess of factor G in the presence of 10 mM GMPPCP at 22° for 10 min in a total volume of 200 μl. The reaction mixture was then layered over 5% sucrose in buffer A and centrifuged in a SW 50.1 rotor at 50,000 rpm (at 4°) for 5 hr. Under these conditions the 50S ribosome–EF-G–GMPPCP complex pellets to the bottom of the centrifuge tube and the excess EF-G is left in the supernatant. The pelleted 50S ribosome–EF-G–GMPPCP complex was taken up in the appropriate buffers for further analysis.

Preparation of Bifunctional Imido Esters. Dimethyl adipimidate, dimethyl suberimidate, and dimethyl sebacimidate were prepared from the corresponding dinitriles as described by Davies and Stark (1969). The imidates were stored over anhydrous $CaCl_2$ in a vacuum desiccator at 2°.

Reaction of the 50S Ribosome–EF-G–GMPPCP Complex with Bifunctional Reagents. The pellets of 50S ribosome–EF-G–GMPPCP complex were taken up in 0.2 M triethanolamine buffer, pH 8.0, containing 10 mM MgAc. The concentration of the ribosomes was maintained at 2–3 mg/ml. The solution of the complex was treated at 2° with bifunctional reagents (~2 mg/ml) for 12 hr. At the end of the reaction, the samples were layered over 5% sucrose in TM containing 1 M NH_4Cl and centrifuged for 5 hr in an SW 50.1 rotor at 50,000 rpm. The pellets contain only the covalently linked G factor; free G factor will be found on the supernatant (Lucas-Lenard and Lipmann, 1971). Sucrose gradients were run on cross-linked preparations showing that intersubunit cross-links do not form under the conditions used.

Sodium Dodecyl Sulfate Gel Electrophoresis. Analyses of the protein aggregates formed as a result of cross-linking experiments were carried out on 5% sodium dodecyl sulfate gels, as described by Weber and Osborn (1969). They were stained with Coomassie Blue after electrophoresis to locate the protein bands.

Chromatography of the Ribosomal Proteins on Agarose Columns. Agarose beads were washed with water and then equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1 mM 2-mercaptoethanol, the buffer used for eluting proteins from agarose. The equilibrated gel was packed to form 1.2 × 30 cm columns. Columns were calibrated by running Blue Dextran, bovine serum albumin, trypsin, and ribonuclease A.

Preparation of ^{3}H-Labeled L7–L12 Protein. The 50S ribosomes were taken up in 0.02 M MgAc–0.01 M Tris-HCl (pH 7.4)–0.001 M 2-mercaptoethanol and L7–L12 protein was extracted by NH_4Cl–ethanol extraction as described by Hamel *et al.* (1972). The L7–L12-deficient 50S ribosomal cores were suspended in a small volume of buffer A, dialyzed against the same buffer, and stored at 2°.

The protein component in the ethanol supernatant was precipitated with 2.25 vol of cold acetone. The precipitate was taken up in starting buffer (6 M urea–3×10^{-2} M methylamine–6×10^{-3} M 2-mercaptoethanol adjusted to pH 5.6 with acetic acid) and dialyzed against the same buffer overnight. The protein was then applied to a CMC column (Moore, 1971) from which L7 and L12, being highly acidic proteins, elute immediately free of their basic contaminants. The L7–L12 fraction was dialyzed against buffer A and stored at 2°.

Reconstitution of 50S Ribosomes with Tritiated L7–L12 Protein. Ethanol–ammonium chloride extracted, nonradioactive ribosomes were incubated with about a twofold excess of tritiated L7–L12 protein for 10 min at 22° in buffer A and then centrifuged through 5% sucrose in TM in an SW 50.1 rotor at 50,000 rpm for 5 hr. The pelleted subunits contained ^{3}H radioactivity consistent with their having been reconstituted.

Ribosomes with ^{3}H label in all proteins except the L7–L12 fraction were made by doing the reverse experiments, *i.e.* by incubating the ethanol–ammonium chloride extracted tritiated ribosomes with cold L7–L12 protein and then isolating the reconstituted 50S ribosomes.

Results

Preparation of EF-G–Ribosome Complexes. Under appropriate ionic conditions, 50S ribosomal subunits, EF-G, and

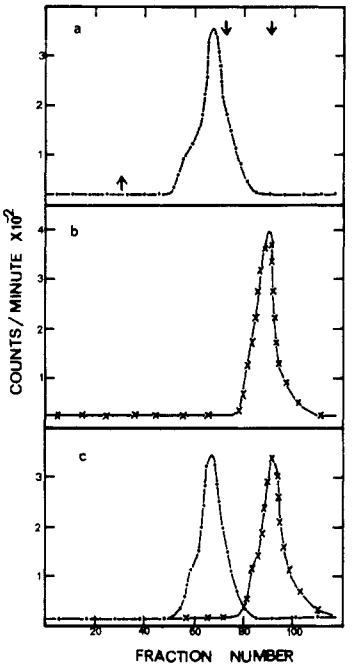

FIGURE 1: Chromatography of (a) ^{35}S-labeled EF-G, (b) tritiated 50S ribosomal proteins, and (c) non-cross-linked EF-G–50S ribosome–GMPPCP complexes on sodium dodecyl sulfate agarose columns. Samples were denatured in 1% sodum dodecyl sulfate in phosphate buffer containing mercaptoethanol by incubating at 40° for 2–3 hr. Five-drop fractions were collected and counted in Brays' solution (Bray, 1960). The lower arrow in frame a indicates the void volume position. The upper arrows indicate the elution positions of bovine serum albumin and lysozyme.

GTP will interact to form a ternary complex which quickly decays as the GTP is hydrolyzed to GDP. If GMPPCP is used in place of GTP, the complex is stabilized because GMPPCP does not hydrolyze. Therefore, in order to form stable ribosome–EF-G complexes for cross-linking, 50S ribosomes were incubated with an excess of GMPPCP and ^{35}S-labeled factor G as described under Materials and Methods. The complexes formed after incubation were separated from unbound EF-G by pelleting through sucrose. The amount of EF-G bound to ribosomes was determined from the radioactivity associated with the ribosomal pellets. Table I summarizes the influence of GMPPCP on the amount of ^{35}S-labeled EF-G recovered in the 50S ribosomal pellet. As expected, GMPPCP strongly stabilizes the 50S ribosome–EF-G complex; the amount of the EF-G pelleted with 50S ribosome increases about fivefold in the presence of the GTP analog. Approximately 40% of the pelleted 50S ribosomes carry bound EF-G in the presence of GMPPCP.

TABLE I: Stabilization of Elongation Factor (EF-G) Binding to 50S Ribosomes by GMPPCP.[a]

Sample	mol of EF-G Bound/mol of 50S Subunits
50S ribosomes	0.075
50S ribosomes + GMPPCP	0.375

[a] 50S ribosomes were mixed with a two- to threefold molar excess of ^{35}S-labeled EF-G in buffer A and incubated with or without GMPPCP at 22° for 10 min. The ribosomes were pelleted through 5% sucrose in TM. The radioactivity per $A_{260m\mu}$ in the pelleted material was measured. Molar ribosome concentrations were estimated from $A_{260m\mu}$ using the extinction coefficient ($E^{1\%}_{260m\mu}$ = 145.0) and particle mol wt (1.7×10^6 daltons) of Hill *et al.* (1969). The molar specific activity of the ^{35}S-labeled EF-G was determined from its $A_{235m\mu}$ (Gordon, 1971) using as its mol wt a value of 80,000 daltons (Kaziro *et al.*, 1969).

TABLE II: Covalent Cross-Linking of EF-G to 50S Ribosomes.[a]

Reaction Mixture	Reagent	High Salt Wash	mol of EF-G Bound/mol of 50 S	% Bound Rel to (1)
(1) Complete	None	−	0.375	100
(2) Complete	None	+	0.038	10
(3) Complete	Glutaraldehyde	+	0.175	46
(4) Complete	Dimethyl sebacimidate	+	0.200	53
(5) Complete	Dimethyl suberimidate	+	0.188	50
(6) Complete	Dimethyl adipimidate	+	0.300	80
(7) Complete	Difluorodinitrobenzene	+	0.313	83

[a] EF-G–50S ribosome–GMPPCP complexes were formed and isolated as described under Materials and Methods. The complexes were exposed to bifunctional reagents (or not) as specified in the table (see Materials and Methods). Non-cross-linked EF-G was removed from the complexes by pelleting through 5% sucrose in TM containing 1 M NH_4Cl.

Reaction of Bifunctional Reagents with the 50S Ribosome–EF-G–GMPPCP Complex. 50S ribosome–EF-G–GMPPCP complexes were taken up in an appropriate buffer and allowed to react with the bifunctional reagents glutaraldehyde, dimethyl sebacimidate, dimethyl suberimidate, dimethyl adipimidate, or difluorodinitrobenzene at 2° for 12 hr. After 12 hr of reaction the complexes were pelleted through 5% sucrose in buffer A containing 1 M NH_4Cl. The washing of ribosomes with buffers containing 1 M NH_4Cl is known to remove noncovalently bound supernatant protein factors associated with ribosomes. Hence any of the ^{35}S counts remaining in the ribosomal pellet should represent the EF-G that has become covalently bound to the 50S ribosomes.

The results of a typical cross-linking experiment are presented in Table II. Most of the ^{35}S-labeled G factor associated with 50S subunits is washed off if the complex is not allowed to react with bifunctional reagents. All of the bifunctional reagents tested were able to form cross-links between EF-G and the 50S ribosome. Dimethyl adipimidate and difluorodinitrobenzene were the most effective.

Analysis of Protein from the 50S Ribosome–EF-G–GMPPCP Complex after Reaction with Bifunctional Reagents. To characterize the nature and extent of cross-linking by these bifunctional reagents, the proteins from the cross-linked complexes were dissociated from ribosomal RNA using sodium dodecyl sulfate and chromatographed on agarose columns in the presence of the detergent. Figure 1a shows the chromato-

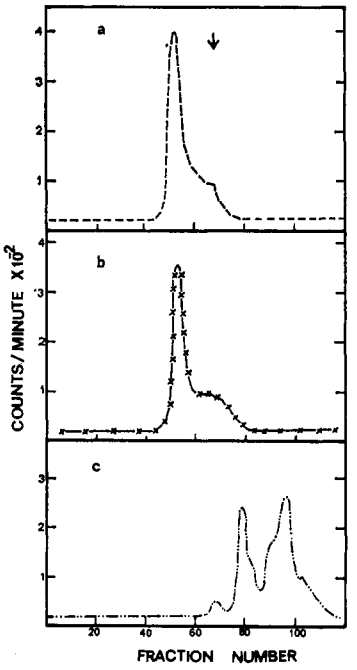

FIGURE 2: Chromatography of proteins from (a) ^{35}S-labeled EF-G–50S ribosome complexes cross-linked with difluorodinitrobenzene; (b) ^{35}S-labeled EF-G–50S Ribosome complexes cross-linked with dimethyl adipimidate; (c) tritiated 50S ribosomes cross-linked with difluorodinitrobenzene in the absence of EF-G. Five-drop fractions collected were counted as described for Figure 1. The arrow in frame a indicates the elution position of free EF-G.

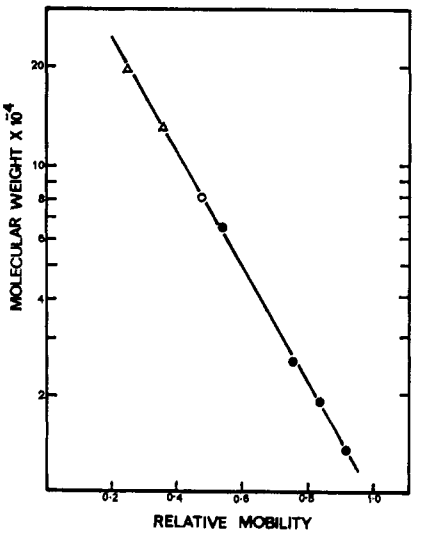

FIGURE 3: Semilog plot of molecular weight *vs.* migration relative to Bromophenol Blue for the EF-G-containing cross-linked ribosomal protein isolated from agarose columns. The standard proteins used were ribonuclease A (mol wt 14,000), lysozyme (17,000), trypsin (25,000), and bovine serum albumin (68,000). EF-G cross-linked to ribosomal proteins was isolated from agarose-sodium dodecyl sulfate columns such as the one used to obtain the results shown in Figure 3. Appropriate fractions were pooled, dialyzed against water, and then lyophilized. The lyophilized material was dissolved in phosphate buffer containing sodium dodecyl sulfate and run on sodium dodecyl sulfate gels according to Weber and Osborn (1969). The location of EF-G in this system is shown (○). It corresponds to a mol wt of 80,000 ± 5000 daltons. The positions of the cross-linked complexes containing EF-G are indicated by triangles (△). The standards are indicated by dots (●).

graphic behavior of a ^{35}S-labeled EF-G preparation used in the present study. The small hump preceding the main peak is probably not a contaminant. It appears to be a G factor aggregate whose yield is dependent on the degree of reduction of the protein. EF-G elutes from these columns a little ahead of bovine serum albumin, as expected for a protein of a mol wt ~80,000 daltons (Kaziro *et al.*, 1969). Figure 1b is a similar elution for ^{3}H-labeled 50S proteins. The majority of the ^{3}H counts elutes at a position close to that of lysozyme (mol wt 17,000 daltons). When proteins from non-cross-linked EF-G–50S ribosome–GMPPCP complexes are chromatographed on agarose columns in the presence of sodium dodecyl sulfate, a profile like that in Figure 1c is obtained. Clearly, in the denatured state, there is little interaction between ribosomal proteins and EF-G. EF-G chromatographs well ahead of the 50S ribosomal proteins.

The chromatographic behavior of the proteins obtained from 50S ribosome–EF-G–GMPPCP complexes reacted with bifunctional reagents is shown in Figure 2. Figure 2a shows the elution properties of ^{35}S-labeled EF-G after it has been cross-linked to 50S ribosomal subunits using difluorodinitrobenzene. The majority of the ^{35}S radioactivity elutes well ahead of the position of uncross-linked EF-G, at a location corresponding to a mol wt of ~200,000. Figure 2b shows the chromatographic behavior of the material obtained from EF-G–ribosome complexes cross-linked with dimethyl adipimidate, which is virtually identical with the profile obtained using suberimidate cross-linking. Thus, it appears that the molecular weight of the EF–G–ribosomal protein complex formed upon massive cross-linking is independent of the cross-linking reagent used to form it.

Figure 2c shows the chromatographic pattern of proteins from tritiated 50S ribosomal proteins cross-linked with difluorodinitrobenzene in the absence of EF-G. It is apparent that the difluorodinitrobenzene cross-linking does not result in the formation of aggretates with molecular weights higher than about 50,000 daltons. Thus, the chromatographic analyses clearly demonstrate the formation of a specific, high molecular weight G factor containing aggregate when the 50S ribosome–EF-G–GMPPCP complex is allowed to react with bifunctional reagents.

The high molecular weight aggregate containing EF-G was isolated from the sodium dodecyl sulfate–agarose column and analyzed by sodium dodecyl sulfate electrophoresis (Figure 3). The high molecular weight material showed two bands upon electrophoresis. The major band ran at a position corresponding to a mol wt of 200,000 ± 20,000. The minor band had an apparent mol wt of 135,000 ± 10,000.

Strategy for Identifying the Ribosomal Components in the Cross-Linked Complex. The major goal in this study is to identify the ribosomal proteins involved in the factor–ribosome interaction. In principle, the amidine linkages formed by the diimidates should be broken by incubation in NH_4OH and acetic acid (Bickel *et al.*, 1972; Wold, 1972). In our hands, however, this procedure was not satisfactory. It appeared to produce peptide bond cleavage and desulfuration, in addition to the cleaving reaction. For this reason efforts were directed toward identifying the ribosomal proteins in the complex by partial reconstitution, using the tritiated 50S ribosomal proteins. 50S ribosomal subunits were reconstituted with specific tritiated 50S ribosomal proteins and the remainder was unlabeled. These subunits were then used to form the cross-linked EF-G–50S ribosome complexes. After cross-linking the products were examined on sodium dodecyl sulfate–agarose columns. The criteria used for deciding whether a given component cross-links to EF-G were: (1) ^{3}H

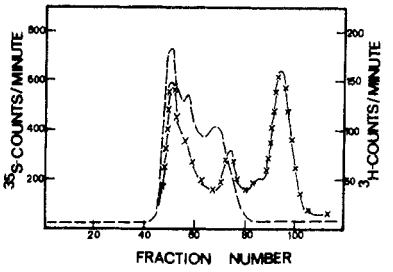

FIGURE 4: Chromatography of proteins from ^{35}S-labeled EF-G–50S ribosome–tritiated L7–L12 complexes cross-linked with difluorodinitrobenzene on agarose columns: 1 pmol of EF-G = 500 cpm of ^{35}S; 1 pmol of L7 = 40 cpm of ^{3}H; (- - -) ^{35}S counts; (X—X) ^{3}H counts.

label due to the component should cochromatograph with ^{35}S-labeled EF-G on agarose columns after cross-linking, and (2) the stoichiometry indicated by the ratio of $^{3}H/^{35}S$ radioactivity in the complex should be an integral molar amount relative to EF-G.

Proteins L7 and L12 Are Required to Form the EF-G–Ribosome–GMPPCP Complex. In view of the results cited above implicating L7 and L12 in the EF-G interaction, it was reasonable to test for the presence of these proteins in the cross-linked complex. Crude ^{3}H-labeled L7–L12 proteins were prepared from tritiated 50S subunits by ethanol precipitation in presence of 0.5 M NH_4Cl. The L7–L12 proteins thus obtained were further purified by chromatography on CMC.

Unlabeled ethanol–NH_4Cl-extracted 50S cores were reconstituted with ^{3}H-labeled L7–L12 proteins as described under Materials and Methods. Both the 50S cores and the reconstituted 50S ribosomal subunits were allowed to complex with EF-G in the presence of GMPPCP. Table III presents data on the amount of ^{35}S-labeled EF-G which binds to native 50S ribosomal subunits, L7–L12 protein deficient cores, and the reconstituted 50S subunits in the presence and absence of GMPPCP. It is clear that L7 and L12 are required to get an EF-G binding which is stable under the conditions used for the isolation of the EF-G complexes, in agreement with other reports (Sander *et al.*, 1972; Hamel *et al.*, 1972).

TABLE III: EF-G Binding to NH_4Cl–Ethanol-Extracted 50S Subunits.[a]

Sample	mol of EF-G Bound/mol of 50S Subunits
50S subunits	0.08
50S subunits + GMPPCP	0.38
NH_4Cl–ethanol-extracted 50S subunits	0.07
NH_4Cl–ethanol-extracted 50S subunits + GMPPCP	0.11
Reconstituted 50S ribosomes	0.09
Reconstituted 50S ribosomes + GMPPCP	0.30

[a] EF-G–50S ribosome complexes were formed and isolated and their EF-G content measured as described for Table I. The preparation of NH_4Cl–ethanol-extracted 50S ribosomes and reconstituted 50S subunits is described under Materials and Methods.

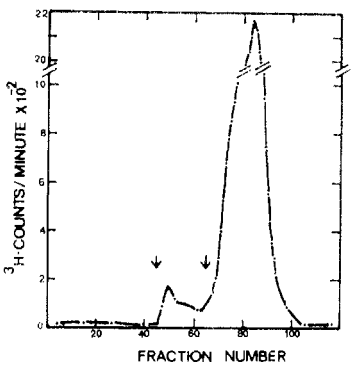

FIGURE 5: Chromatography of proteins from ^{35}S-labeled EF-G–50S ribosome complexes (non-L7–L12 proteins are tritiated) cross-linked with difluorodinitrobenzene. The profile is given with respect to ^{3}H alone. Aliquots (2 μl) from each fraction were counted. The fractions from high molecular weight regions (>80,000 daltons) were pooled (as indicated by the arrows) and the material was isolated after dialysis by lyophilization.

Our observation that EF-G will not bind to 50S ribosomes in the presence of GMPPCP in the absence of L7 and L12 is at odds with the recent report of Hamel and Nakamoto (1972). They found no requirement for L7 and L12 for the GMPPCP-stablized binding. Our results, reported in Table III, however, agree with those of Sander *et al.* (1972). The source of these inconsistencies is not clear to us at this time.

Proteins L7–L12 Will Cross-Link to EF-G. Complexes of EF-G–GMPPCP–50S ribosomes with tritiated L7–L12 protein were cross-linked with difluorodinitrobenzene, and the cross-linked product was isolated by centrifugation as described earlier. The results of chromatographic analysis of the protein isolated from such cross-linked 50S ribosomes on sodium dodecyl sulfate–agarose column are shown in Figure 4. As a result of cross-linking the EF-G–50S ribosome complex, a substantial amount of ^{3}H label (namely L7–L12) cochromatographs with the ^{35}S label (EF-G) in the 180,000–200,000 dalton region. The ratio of ^{3}H to ^{35}S in the 200,000 dalton product points to a stoichiometry of about 2.0–2.5 equiv of L7–L12 protein per EF-G. This result is consistent with the observation that there are about two–three copies of L7–L12 protein on the 50S ribosomes (Deusser, 1972; Weber, 1972). EF-G and L7–L12 protein together account for about 110,000 daltons of the total mol wt of 200,000 ± 20,000 daltons in the cross-linked product.

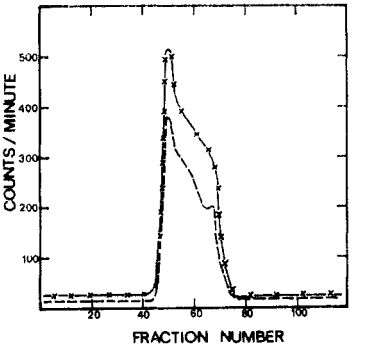

FIGURE 6: Rechromatography of the high molecular weight product (Figure 5) on agarose columns. The specific activity of EF-G is about 500 cpm/pmol. The specific activity of tritiated proteins for 1 pmol of protein of 10,000 daltons = 60 cpm: (- - -) ^{35}S counts; (X—X) ^{3}H counts.

Amount of Proteins Other Than L7 and L12 Present in the Cross-Linked EF-G Complex. To ensure that the accounting of the proteins in the cross-linked product given above is correct, the reverse of the experiment just described above was carried out. Reconstituted 50S ribosomes formed from ^{3}H-labeled 50S cores and cold L7 and L12 were complexed with ^{35}S-labeled EF-G and cross-linked with difluorodinitrobenzene. The proteins from the cross-linked ribosomes were analyzed on sodium dodecyl sulfate–agarose columns (Figure 5). The amount of protein (^{3}H counts) eluting in the region of the profile representing mol wt 120,000–200,000 material accounts for about 6% of the total ^{3}H counts from the ribosomes. One would expect nearly 100,000 daltons of ribosomal protein in the EF-G complex in addition to L7 and L12. Since only about 40% of the ribosomes bind EF-G in our hands, and the cross-linking is about 70–80% effective, the amount of ^{3}H expected in the high molecular weight region of the elution profile is about 5.5%. The material eluting in this region was isolated and rechromatographed on the same column. The elution profile of the high molecular weight product (higher than 80,000) is shown in Figure 6. The ratio of ^{3}H to ^{35}S on the leading side of the ^{35}S peak is consistent with there being about 80,000–90,000 daltons of ^{3}H-labeled non-L7–L12 protein per EF-G. Thus, the probable composition of the complex is one EF-G molecule (80,000 daltons) plus two molecules of L7–L12 protein (26,000 daltons) plus 80,000–90,000 daltons of non-L7–L12 50S protein, making a total mol wt of 186,000–196,000 daltons, consistent with the molecular weight observed.

The ratio of ^{3}H to ^{35}S on the low molecular weight side of the ^{35}S peak in Figure 6 increases. The ratios of ^{3}H to ^{35}S in this region imply aggregate molecular weights too high for the elution location, if one assumes that the tritiated protein and ^{35}S-labeled EF-G are cross-linked. Accordingly there must be cross-linked ribosomal protein eluting in this region independent of EF-G. In total, the non-EF-G associated protein eluting with a high molecular weight amounts to no more than about 5000 daltons per 50S subunit, a very low molar yield. It is interesting to note that no 50S protein elutes in this region when cross-linking is done in the absence of EF-G.

Discussion

EF-G is readily cross-linked to the ribosome by bifunctional protein reagents. Of the five reagents tested, dimethyl adipimidate and difluorodinitrobenzene appear to be the best for this purpose. The fully extended length of the dimethyl adipimidate cross-link is about 8.6 Å (Hartman and Wold, 1967) and the length of bridge formed using difluorodinitrobenzene is about 6 Å (Marfey *et al.*, 1965a,b). What we observed is that as the cross-link length increased past 10 Å, the efficiency with which EF-G became fixed to ribosomes decreased markedly.

Extensive reaction with dimethyl adipimidate and difluorodinitrobenzene will cross-link about 120,000 daltons of 50S ribosomal protein to each bound G factor molecule. Approximately 30,000 daltons of cross-linked protein is accounted for by components L7 and L12 of the 50S subunit, both of which have been amply implicated in the EF-G–ribosome interaction. Thus both components must be located close to EF-G when it is bound to the ribosome.

Work is going forward to establish the identity of the remaining 90,000 daltons of ribosomal protein in the cross-linked complex. This material could represent as many as six–eight ribosomal components or as few as three–four. The presence of these components in the complex raises the question of whether proteins L7–L12 actually contact EF-G or instead cross-link to it through these other components. Preliminary data indicate that the approximately 100,000 dalton (EF-G) containing product observed on sodium dodecyl sulfate gels of cross-linked ribosomes, which has been occasionally seen on agarose columns as well, includes the proteins L7–L12. The occurrence of such structures would appear to indicate that direct links can form between EF-G and proteins L7–L12. Further effort, however, will be required to document this point in a satisfactory way.

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Rat Ventral Prostate Chromatin. Effect of Androgens on Its Chemical Composition, Physical Properties, and Template Activity†

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ABSTRACT: The effect of castration and hormone replacement on the chemical composition, physical properties, and template activity for *Escherichia coli* RNA polymerase of rat ventral prostate chromatin was examined. Chromatin isolated by two methods, one with and the other without intentional shearing, from normal rats (N), 3-day castrates (C), and castrates treated with testosterone propionate for either 4 (CT_4) or 72 (CT_{72}) hr, was compared. Chromatin solubilized by mechanical shearing contained four major fractions, but when intentional shearing was avoided, it included two major components, as judged by analytical ultracentrifugation and sucrose gradient centrifugation. Hormone-dependent increases in protein and RNA content occurred within the first 4 hr of androgen administration. Paradoxically the template activity of prostate chromatin from normal rats or castrated rats treated for 3 days with testosterone propionate was less than that from castrates. However, chromatin from 3-day castrates treated for 4 hr with androgen was 80% more active than C chromatin. These results did not depend upon demonstrable differences in the activities of nucleases, histone protease, or ribonucleoside triphosphatase. The marked increase in nonhistone protein content and stimulation of template activity following brief exposure to androgens may reflect hormone-induced changes in the structure of prostate chromatin.

Many cellular constituents and enzymatic activities of male accessory reproductive glands are altered by changes in the level of circulating androgens. In the prostate, castration leads to decreased protein and RNA synthesis, and subsequent involution of cytoplasmic structures (Price and Williams-Ashman, 1961). Hormone-dependent changes in the macromolecular composition of prostate cytoplasm and nuclei (Liao, 1965; Anderson *et al.*, 1970; Chung and Coffey, 1971) and the effect of androgens on prostatic RNA synthesis, both *in vivo* (Fujii and Vilee, 1968, 1969) and *in vitro* (Liao *et al.*, 1965; Liao and Fang, 1969), have been described.

Two hours after a subcutaneous injection of testosterone propionate to rats castrated for 70 hr, RNA synthesis in isolated prostate nuclei, incubated under conditions of low ionic strength, increased 40%. The formation of ribosomal RNA or its precursors was particularly stimulated (Liao *et al.*, 1966).

When the template activity of rat ventral prostate chromatin from pressure-disrupted nuclei was assayed with excess *Micrococcus lysodeikticus* RNA polymerase, no consistent difference was observed between chromatin isolated from 3-day castrates and that from castrates treated with testosterone propionate for 3 days (Liao and Lin, 1967). Compared to the endogenous RNA polymerase activity of intact nuclei, chromatin template activity was about ten times greater, nearest neighbor frequency analysis of the newly synthesized RNA differed markedly, and binding of actinomycin D was increased. These results suggested that the bacterial enzyme utilized template sites that were not transcribed by the endogenous RNA polymerase of isolated nuclei.

Mangan and coworkers compared the template activity of isolated prostate nuclei and prostate chromatin (Mangan *et al.*, 1968). The template activity for *Escherichia coli* polymerase of prostate nuclei from testosterone-treated rats exceeded the activity of nuclei from castrates, but chromatin template activity exhibited no consistent differences. Mainwaring *et al.* (1971) observed that 4 hr after administration of androgens, prostate chromatin, assayed under conditions of low ionic strength, exhibited an increase in template activity of about 10%.

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