IDENTIFICATION OF NEIGHBOURING PROTEINS IN THE 30 S RIBOSOMES OF *E. COLI*

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1. Introduction

Bifunctional reagents have been widely used recently in studies of neighbourhood relations between proteins of *E. coli* ribosomes [1–9]. The composition of most of the cross-linked protein complexes so far observed after treatment of 30 S ribosomes with these reagents are consistent with proximity relationships implicit in the 30 S assembly map [10], with the protein compositions of ribonucleoprotein fragments isolated after ribonuclease digestion of 30 S ribosomes [11–13] and with protein neighbourhoods deduced from analyses of functional sites in 30 S ribosomes [14]. The possibility that cross-linked complexes might be formed in some cases as a consequence of structural deformation of ribosomes in the presence of bifunctional reagents seems unlikely because it has been shown that functionally active 30 S subunits can be reconstituted in vitro from 16 S rRNA and protein mixtures in which 30 S proteins S 5 and S 8 are replaced by a dimethyl adipimidate cross-linked complex of S 5 and S 8 [15].

Two major difficulties are encountered in the use of bifunctional reagents for the study of ribosome structure; isolation of pure complexes from the mixture of reaction products and the identification of the proteins present in isolated complexes. Here we describe briefly experimental techniques which overcome these difficulties and their application to the study of *E. coli* 30 S ribosome subunits.

2. Materials and methods

2.1. *Buffers*

1) 0.1 M triethanolamine–HCl, pH 7.2, 0.01 M $MgCl_2$, 0.4 M NaCl, 0.006 M β-mercaptoethanol. 2) 0.1 M triethanolamine–HCl, pH 7.2, 0.01 M $MgCl_2$, 0.05 M KCl, 0.006 M β-mercaptoethanol 3) 2 M triethanolamine-base, 0.05 M KCl, 0.01 M $MgCl_2$. 4) 0.01 M triethanolamine–HCl, pH 7.2, 0.5% SDS, 0.01 M β-mercaptoethanol. 5) 0.1 M triethanolamine–HCl, pH 7.2, 0.01 M $MgCl_2$.

2.2. *Preparation of ribosomes and ribosomes subunits*

^{35}S-labelled ribosomes prepared as previously described [6,7] are dissociated by centrifugation for 18 hr at 20 000 rev/min, 4°C in a SW 25.1 Spinco rotor 5–20% sucrose gradients prepared in buffer 1 and dissociated subunits are concentrated by precipitation with 10% polyethylene glycol [16] resuspended at a concentration of 10 0D at 260 nm/ml in buffer 2 and stored at –20°C. The specific activity of ^{35}S-labelled 30 S subunits is from 2 to 5 × 10^6 cpm/0D at 260 nm.

2.3. *Reaction of ribosomes with dimethyl suberimidate*

Dimethyl suberimidate hydrochloride (DMS) prepared as described by Davies and Stark [17] and stored at 4°C in a dessicator is dissolved immediately before use at a concentration of 40 mg/ml in a mixture of buffer 5, and 2 N KOH (85:15 v/v). The pH of a suspension of 30 S ribosomes (10 0D 260/ml) in buffer 2 is adjusted to pH 8.2 by addition of 0.06 vol of buffer 3, 0.1 vol of the solution of DMS is then added (final DMS concentration : 4 mg/ml) and the mixture is incubated at 30°C for 90 min. Ribosomes are precipitated from reaction mixtures by addition of 0.6 vol of ethanol recovered by centrifugation, resuspended in buffer 2, and the proteins are extracted by the acetic procedure [18,19] concentrated from acetic acid solution by addition of 5 vol of acetone

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[20] collected by centrifugation, redissolved in a small volume of 8 M urea and reprecipitated with acetone to remove residual acetic acid. Final protein precipitates are dissolved in 8 M urea, 0.1 M β-mercaptoethanol at a concentration of 30–50 mg/ml.

2.4. *2D Electrophoresis on polyacrylamide*

Samples of proteins (10 μl, 300 μg total protein) prepared as described above from DMS treated 30 S ribosomes are subjected to electrophoresis in two dimensions using the buffer system of Kaltschmidt and Wittmann [21] and a reduced scale apparatus in which the second dimension electrophoresis is carried out on 140 mm × 120 mm × 1 mm thick horizontal gel slabs. Second dimension gel slabs contain 0.25% bisacrylamide. This apparatus and its use are described elsewhere [22]. After electrophoresis gels are stained with Coomassie Brilliant Bleu R and dried for autoradiography.

2.5. *Elution and ammonolyse of the complexes*

Stained spots corresponding to crosslinked complexes are cut out of the dried gel slabs and their ^{35}S content is quantitatively eluted by incubation for 48 hr at 37°C in 1 ml of buffer 4. ^{35}S-labelled material is recovered from the remainder by addition of 300 μg of unlabelled carrier 30 S proteins followed by five vol of acetone [30]. Precipitated proteins are collected by centrifugation, redissolved in 200 μl of 10% SDS 0.2 M β-mercaptoethanol, a small sample (2–5000 cpm) is removed for use in molecular weight determinations, 800 μl of 6.5 M NH_4OH, 1.0 M acetic acid is added and the mixtures are incubated for 18 hr at 30°C to cleave DMS cross-links.

2.6. *Analysis of ammonolysis products*

After ammonolysis as described above reaction products are precipitated by addition of five vol of acetone to incubation mixtures, and collected by centrifugation. Pellets are washed several times with acetone, placed briefly in vacuo, dissolved in 10 μl of 8 M urea, 0.1 M β-mercaptoethanol and fractionated by 2D gel electrophoresis using second dimension gel slabs containing 0.5% bisacrylamide (21). Gel slabs are stained, dried and autoradiographed (22) and spots corresponding to each of the 30 S ribosomal proteins and to products visible on the autoradiograph are cut out and dissolved by incubation at 50°C overnight in 50% H_2O_2 (0.2ml) in sealed vials. Radioactivity is then measured after addition of 1.5 ml of toluene–Triton X 100 scintillation fluid.

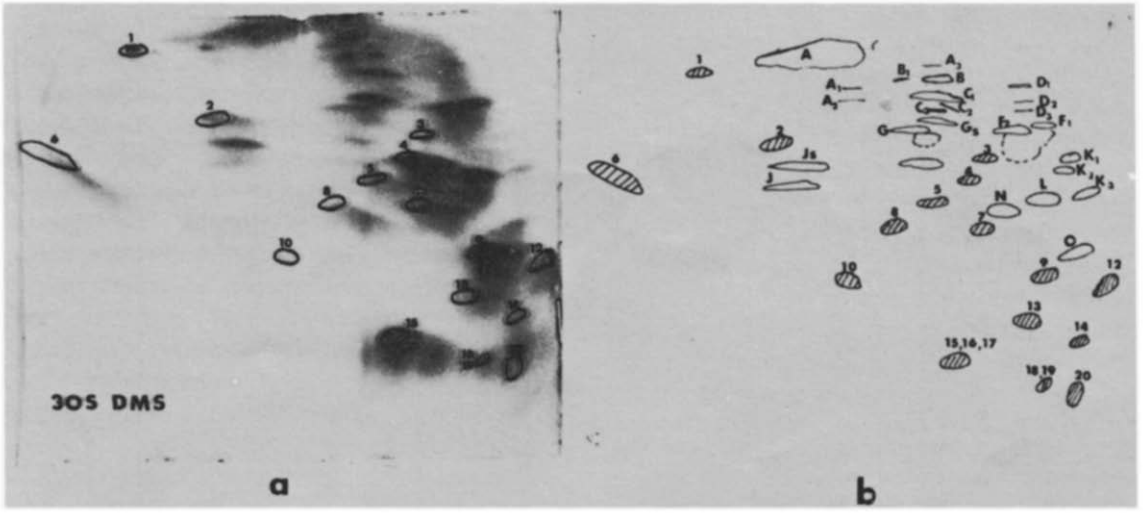
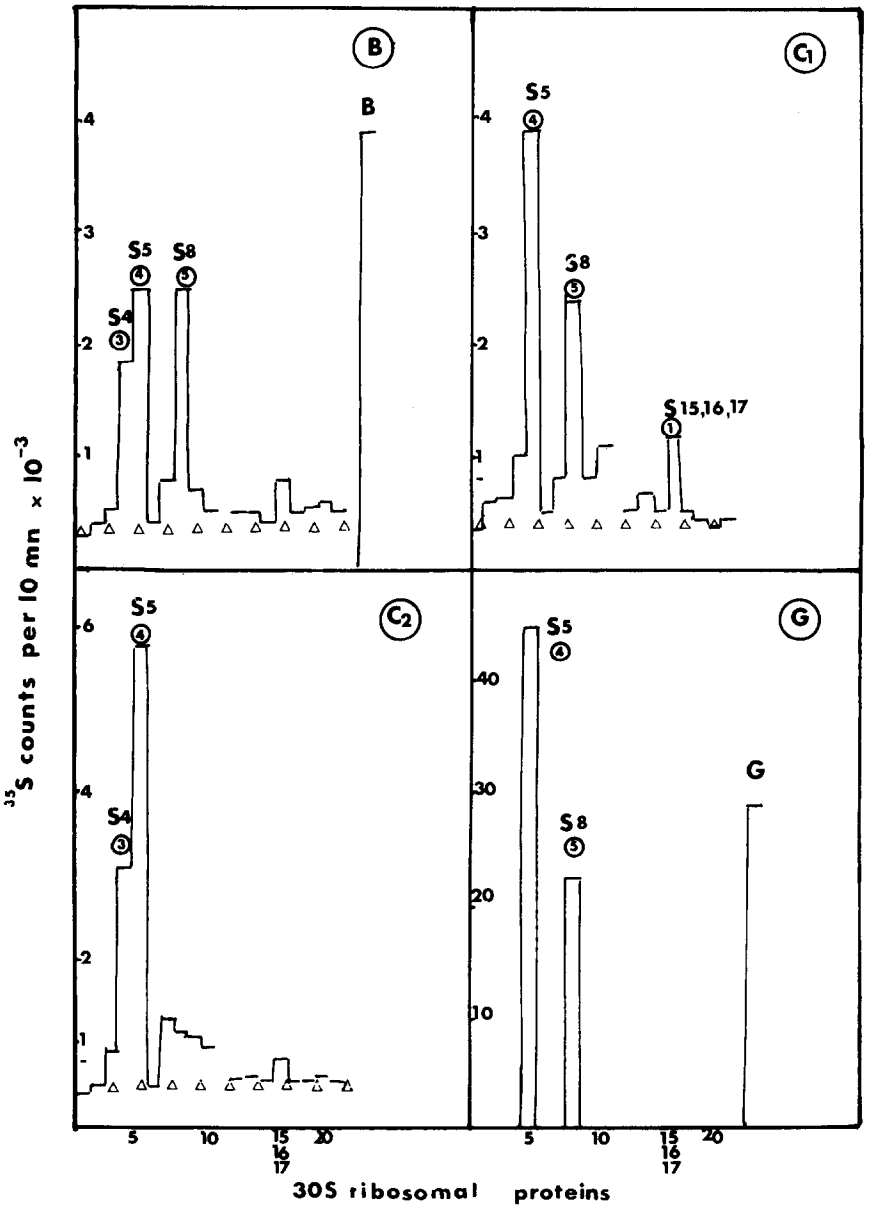


Fig.1. a) Distribution of stained regions and radioactivity in a 2D gel upon which a mixture of proteins prepared from DMS-treated unlabelled and ^{35}S labelled untreated 30 S ribosome subunits has been analysed (Materials and methods). Positions of control ^{35}S-labelled proteins detected by autoradiography of the stained dried gel have been drawn on the photograph of the gel. b) Schematic drawing showing the positions of extra spots in 2D gels of proteins of DMS-treated 30 S ribosomes. The laboratory nomenclature of these spots is given and the positions of some control 30 S proteins are shown for comparison purposes.

2.7. *Determination of molecular weights of cross-linked complexes*

Samples of DMS cross-linked complexes are submitted to electrophoresis in the presence of SDS according to the procedure described by Laemmli [23] using a mixture of monomeric, dimeric, trimeric and tetrameric species produced by DMS treatment of haemoglobin as molecular weight standards. Electrophoresis is carried out in a vertical 1 mm thick gel slab. After electrophoresis, gels are stained, dried and autoradiographed. Molecular weights of radioactive bands (complexes) detected by autoradiography are calculated by reference to the stained haemoglobin oligomer bands.



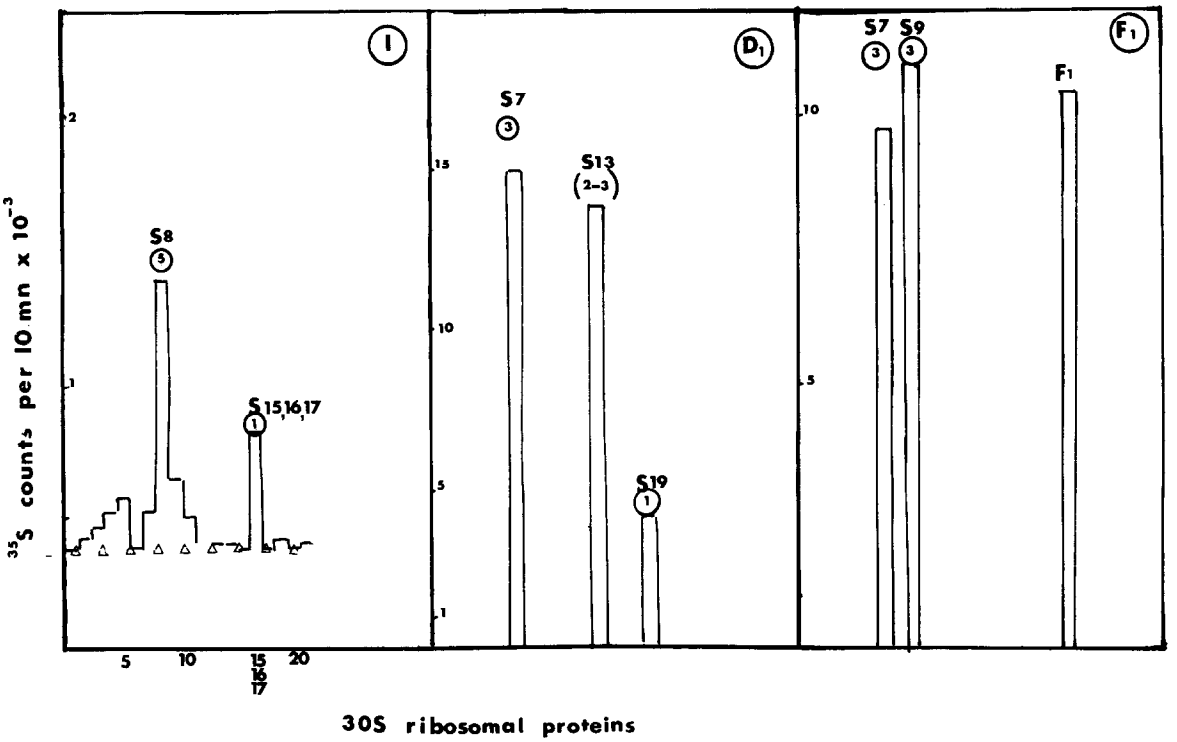


Fig.2. Histograms of radioactivity distribution in 2D gels upon which ammonolysis products of DMS complexes have been analysed. Spots corresponding to all 30 S proteins were cut out of the dried 2D gels and their radioactivity was counted. Radioactivity measured at the position of the original complex is noted for complexes B, G and F1. Fig.2a: Complexes B, C1, C2, G fig.2b: Complexes I, D, F (△—△—△) counter background (O) sulfur content.

3. Results

3.1. *Alteration of ribosomal protein electrophoretic patterns induced by dimethyl suberimidate*

The patterns of stained and radioactive spots obtained by 2D electrophoresis of a mixture containing proteins isolated from labelled DMS treated 30 S ribosomes and a trace of ^{35}S labelled 30 S proteins is shown in fig.1. These patterns are obtained reproducibly with independant ribosomes preparations and although some regions of gel slabs shown a significant background stain a considerable number of spots corresponding to material migrating to positions different from those occupied by normal 30 S ribosomal proteins are readily detected. Those among them which migrate more slowly than any 30 S protein probably correspond to cross-linked complexes containing two or more proteins. Others with higher migration rates in the second dimension of electrophoresis may contain cross-linked complexes but can also correspond to single ribosomal proteins whose electrophoretic properties are modified by the presence of intramolecular DMS cross-links or of DMS residues which have reacted at one end with a protein NH_2 group and at the other with a solvent molecule. Fig.1b is a drawing which gives the laboratory nomenclature of the new spots and shows their approximate positions and those of some normal 30 S proteins in 2D gels.

3.2. *Identification of protein components and determination of the molecular weights of isolated cross-linked complexes*

In order to identify their protein components cross-linked complexes separated by 2D electrophoresis as described above are isolated by elution of appropriate regions of dried gel slabs and cleaved by ammonolysis.

Table 1

Complex	Proteins	P. M. $\times 10^{-3}$	P. M. measured $\times 10^{-3}$	CP/10MN/S³ Background deduced
B	S 4 3 S 5 4 S 8 5	61.8	59	500 500 400
C1	S 5 4 S 8 5 S 15 16 1 17	46 47.6	46	850 400 700
C2	S 4 3 S 5 4		46	700 1300
G	S 5 4 S 8 5	35	34	11 000 4200
I	S 8 5 S 15 16 1 17	28	26 000	200 500
F	S 7 3 S 9 3	39	35 000	3000 3500
D1	S 7 3 S 13 2–3 S 19 1	50		4800 4500–6700 4000

Calculated and measured mol. wt ^{35}S contents and protein compositions of DMS cross-linked complexes of 30 S proteins.

Using the condition described here the yield of the cleavage reaction is about 70%. Longer incubation times cause modification of the electrophoretic properties of the released proteins. Identification of the ammonolysis products is achieved by 2D electrophoresis in the presence of carrier 30 S proteins. Histograms of the radioactivity distributions measured in 2D gels upon which the ammonolysis products of complexes B, C1, C2, G, I, D1, F1, (fig.1b) had been fractionated are shown in fig.2. Complementary data needed for unambiguous interpretation of these results is obtained by measurement of the approximate molecular weights of the isolated cross-linked complexes (Materials and methods) and by the calculation, based on the published molecular weights and methionine and cysteine contents of 30 S proteins [24–26], of their sulfur contents. Table 1 summarizes results derived from the histograms in fig.3 and from molecular weight and sulfur content estimations, and lists the radioactivity found per sulfur atom in the various proteins identified in each complex, and the compositions deduced for these complexes.

4. Discussion

The methods described here permit the separation of large numbers of cross-linked complexes from the products of the action of bifunctional reagents on ribosomes, the isolation of these complexes in a pure state, and when bisimidoesters are used as cross-linking agents, the cleavage of isolated complexes in good yield. The products of ammonolysis of cross-linked complexes show the same electrophoretic behaviour as control ribosomal proteins and can be readily identified by 2D electrophoresis. Estimation of the molar ratios of identified proteins in cross-linked complexes depends on the accuracy with which the sulfur contents

of these proteins can be calculated and on the assumption that uniform labelling of ribosomal protein is achieved by growth of bacteria in the presence of ^{35}S under the conditions we use [7]. Control experiments to be described elsewhere have shown that this assumption is valid and we consider that the calculated sulfur contents of 30 S proteins are accurate to within at least ± 1 S atom per molecule. This conclusion is supported by results in the table which show that with the exception of protein S 8 approximately the same amount of radioactivity is found per sulfur atom in each protein of a complex. The small amounts of radioactivity found in protein S 8 (complexes C1, B1, G, I; table) are due to experimental losses. S8 has a very low mobility in the first dimension of electrophoresis (see fig.1a) and under the conditions we use, in which the initial protein sample is not polymerised any fraction of this protein which does not enter the first dimension gel will be lost during dialyses of this gel prior to its incorporation in the second dimension gel slabs. The composition of the series of complexes: B, C1, C2, G, I suggest that the proteins which they contain are arranged in the order S 4–S 5–S 8–S $15_{/16/17}$ in 30 S ribosome subunits since no protein pair such as S 4–S 8, S 4–S $15_{/16/17}$ or S 5–S $15_{/16/17}$ has yet been identified in preliminary analyses of other cross-linked complexes. Although the compositions of complexes C1 and I have not so far been established unambiguously because the separation of proteins S 15, S 16 and S 17 is incomplete in our 2D gels, comparison of dried gels and their autoradiographs indicates that the unidentified component of these complexes is probably S 15.

A considerable number of complexes in addition to those whose caracterisation is described here can be detected in the protein moiety of DMS treated 30 S ribosomes. In addition the use of other bisimidoesters as cross-linking agents, e.g. dimethyl adipimidate (DMA), succinimidate (DMSuc) and Sebacimidate (DMSeb) produces analogous but not identical families of complexes and application of these reagents to the study of the structure of the 50 S ribosome subunit gives results comparable to those described here for the 30 S subunit. The characterisation of many of these complexes is in progress and the results obtained together with a detailed account of the experimental procedures described briefly here will be reported elsewhere.

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