IDENTIFICATION OF NEIGHBOURING PROTEINS IN *E. COLI* 50S RIBOSOMES

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1. Introduction

Several recent reports have described the use of protein specific bifunctional reagents for the identification of neighbouring proteins in *E. coli* 30S ribosomes. Components of cross-linked complexes isolated from 30S ribosomes have been identified either by the analysis of the reaction of the complexes with antibodies against individual 30S ribosomal proteins or by cleavage of cross-links and identification of cleavage products by polyacrylamide gel electrophoresis [1–8]. We have previously reported preliminary results obtained by the application of these techniques to the study of protein neighbourhood relationships in *E. coli* 50S ribosomes [9,10]. Here we describe results obtained in further experiments in which we have identified the components of several cross-linked complexes isolated from 50S ribosomes treated with dimethyl suberimidate.

2. Materials and methods

Materials and preparative and analytical procedures are as previously described [11] with the exception of mol. wt determination by electrophoresis in SDS containing polyacrylamide gels which is carried out as follows. Small aliquots (2000–5000 cpm ^{35}S, 5–50 μl) of eluates of spots containing cross-linked complexes cut out of dried gel slabs of the type shown in fig.1A (for details consult [11]) are submitted to electrophoresis in SDS containing polyacrylamide gels using the procedure described by Laemmli [12]. Haemoglobin (mixed Hb, Hb_2, and Hb_4) is used as a mol. wt standard. After electrophoresis gel slabs are dried and stained to reveal haemoglobin and its dimer and tetramer and radioactive bands are detected by autoradiography. The electrophoretic mobilities of Hb, Hb_2 and Hb_4 are found to be directly proportional to the

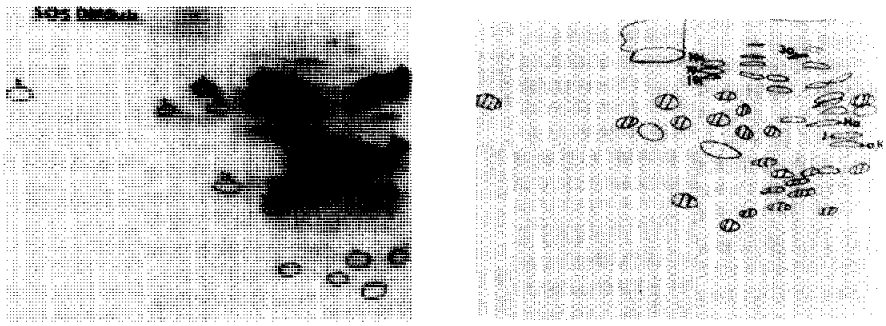

Fig.1. Effect of DMS treatment on the electrophoretic properties of *E. coli* 50S ribosomal proteins. (A) Photograph of a stained, dried gel slab in which a mixture of DMS treated unlabelled, and control ^{35}S-labelled *E. coli* 50S ribosomal proteins were analysed. The positions of the control proteins detected by autoradiography have been drawn onto the photograph. (B) Drawing showing the positions and nomenclature of some of the new protein species formed by DMS treatment of 50S ribosomes. The positions of a number of normal 50S proteins (hatched spots) are indicated for comparison purposes.

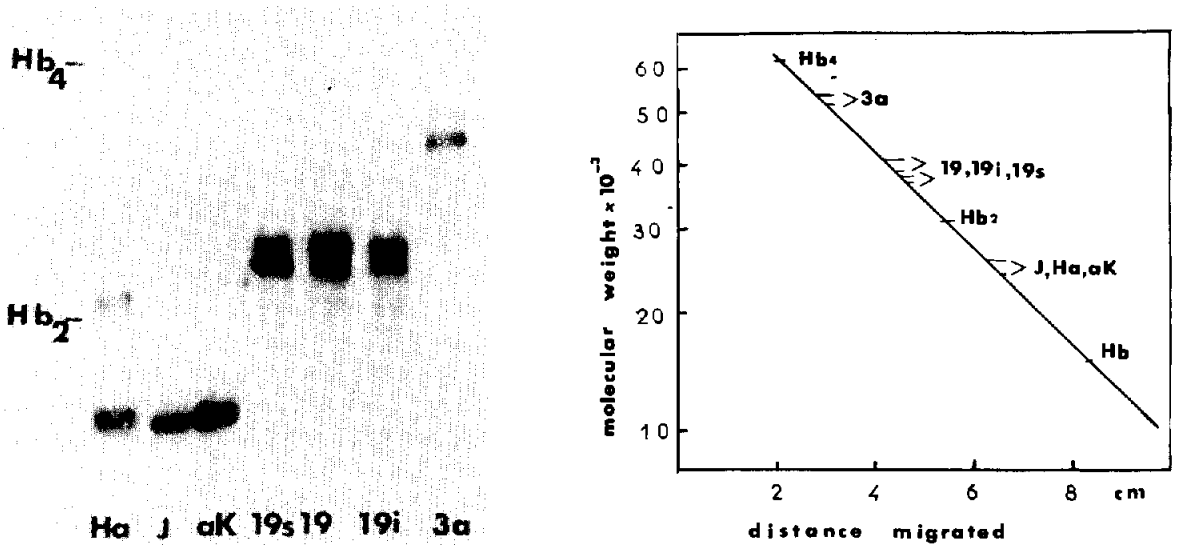

Fig.2. Homogeneity and mol. wts of complexes 3a, j, Ha, aK, 19, 19S, and 19i. (A) Autoradiograph of a gel slab in which complexes 3a, j, Ha, aK, 19, 19S, 19i were subjected to one dimensional electrophoresis in the presence of SDS. The position of Hb2 and Hb4 reference bands are indicated. (B) The distances migrated by Hb, Hb2 and Hb4 in the gel slab whose autoradiograph is shown in fig.2A are plotted against their mol. wts (log scale). The distances migrated by complexes 3a, Ha (major component) j, aK and 19, 19S 19i, (both components) and the estimated mol. wts of these products are indicated.

logarithms of their mol. wts (fig.2). Assuming that the same relationship holds for DMS cross-linked 50S ribosomal protein complexes the mol. wts of these products can then be estimated.

3. Results

3.1. *Alteration of 50S ribosomal protein electrophoretic patterns induced by treatment with dimethyl suberimidate (DMS)*

As noted in our previous report concerning 30S ribosomes treatment of 50S ribosomes with DMS causes a drastic modification of the electrophoretic behaviour of their proteins. Patterns of stained and radioactive spots obtained by two-dimensional electrophoresis of a mixture containing proteins isolated from DMS treated unlabelled 50S ribosomes and a trace amount of ^{35}S-labelled untreated 50S proteins are shown in fig.1. Stained spots (DMS treated proteins) are more elongated and migrate as slightly more basic products than the radioactive spots (unmodified proteins). A considerable number of stained products which migrate to positions different from those occupied by normal 50S proteins can be seen in fig.1. Products of this kind with low second dimension electrophoretic mobilities probably correspond to cross-linked complexes containing two or more 50S proteins. Others with higher mobilities in the second dimension of electrophoresis may be cross-linked complexes but may correspond to single 50S 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. In spite of the presence of a diffuse stained background in several regions of gel slabs of the type shown in fig.1 most of the stained spots generated by DMS treatment are well defined and can be detected reproducibly in different cross-linked protein preparations. We have however noted that the relative intensities of some of these DMS generated spots can vary from one protein sample to another and as a function of the age of cross-linking reagent preparations.

3.2. *Characterisation of complexes Ha, j, aK, 19S, 19, 19i, and 3a*

3.2.1. Homogeneity and molecular weight. Fig.2A shows the results of electrophoretic analysis of material

eluted from spots Ha, j, aK, 19S, 19, 19i and 3a. Complexes 3a, j, and aK migrate as single products, Ha contains two components, one present in much larger amount than the other, and complexes 19, 19i and 19S each contain approx. equal amounts of two components with almost identical electrophoretic mobilities. Estimates of the mol. wts of these products are made in fig.2B.

3.2.2. Protein composition. The products of ammonolysis of DMS cross-linked ribosomal protein complexes under the conditions used in the experiments described here (for full details see [11]) contain about 30% of unreacted complex and 70% of cleavage products. When the ammonolysis products are analysed by two-dimensional acrylamide gel electrophoresis three components are therefore observed.One has the electrophoretic properties of the original complex and the other two display altered first and second dimension electrophoretic mobilities. The results of two-dimensional analyses of the products of ammonolysis of complexes 3a, 19, and aK are summarised in fig.3 in which the upper and lower parts are respectively reproductions of autoradiographs of stained and dried gel slabs, and drawings showing the distribution of stained spots in the same slabs. Three radioactive spots can be seen in the upper panel of each part of fig.3, and can be identified as follows by comparison with the results shown in the lower panels and with the known electrophoretic mobilities of the cross-linked complexes subjected to ammonolysis: fig.3a, residual complex 3a and 50s proteins L2 and L9; fig.3b, residual complex 19 and 50S proteins L10 and L11; fig.3c, residual complex aK and 50S proteins L17 and L32.

Since the stained spots whose positions are indicated in the lower panels of fig.3 correspond to carrier 50S

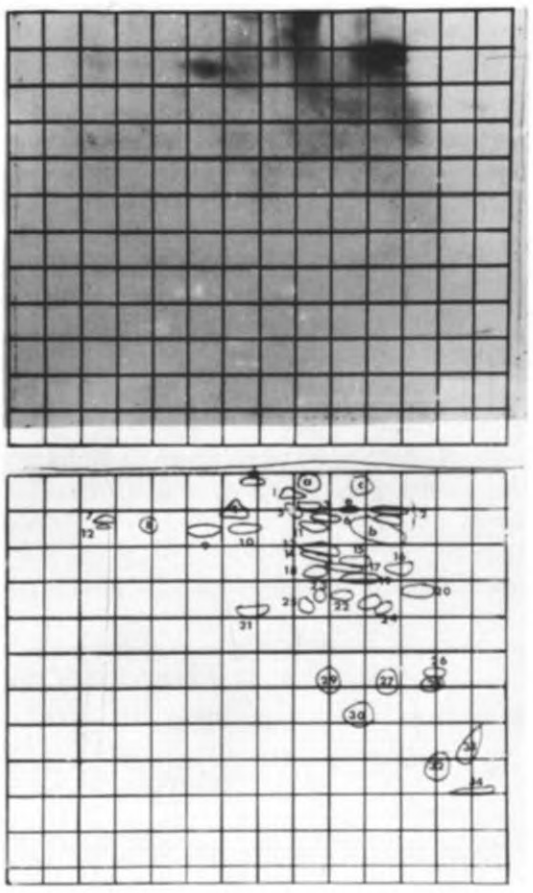

Fig.3a

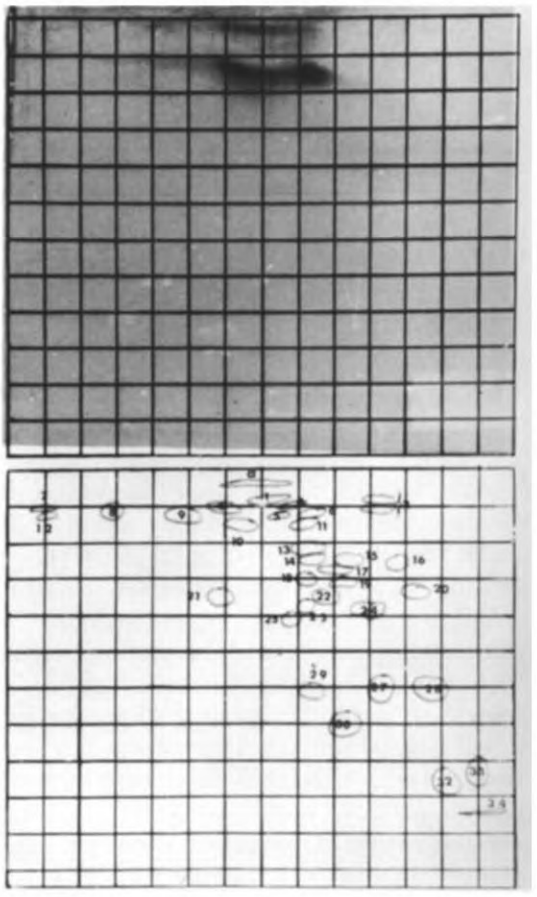

Fig.3b

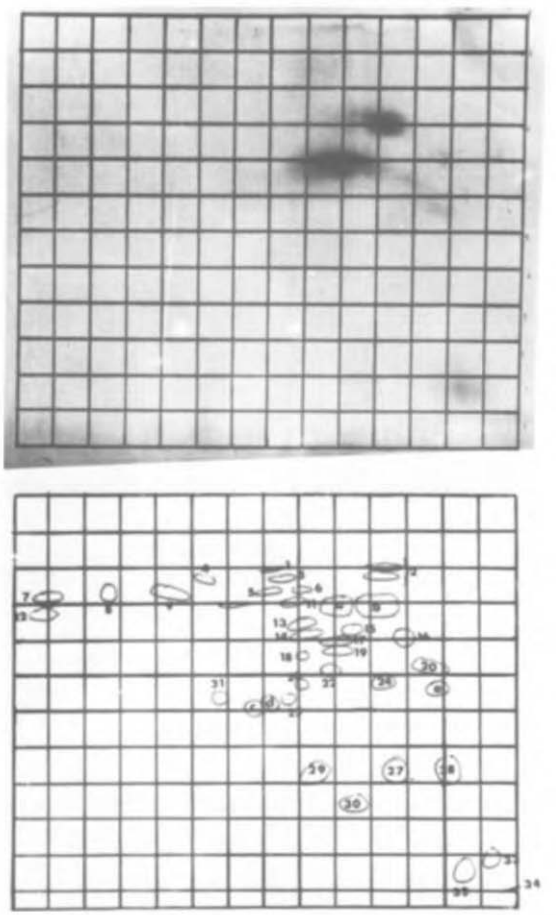

Fig.3. Identification of cleavage products of complexes 3a, 19 and aK Upper panels: autoradiographs of gel slabs upon which the cleavage products of ^{35}S-labelled complexes 3a, 19 and aK mixed with unlabelled control 50S proteins were separated by two dimensional electrophoresis. Lower panels: drawings showing the distributions of stained spots (control proteins) in the gel slabs whose autoradiographs are presented in the upper panels. A graticule is superimposed on each panel to facilitate the comparison of the positions of radioactive and stained spots.

proteins which have been subjected to the treatment used to cleave DMS cross-links (incubation for 15 h at 30°C in 6.5 M NH_4OH, 1.0 M acetic acid) the validity of these identifications depends on the demonstration that such treatment does not significantly alter the electrophoretic behaviour of 50S proteins. Proof that this is the case will be presented elsewhere but it may be noted that comparison of the distributions of stained spots in the lower panels of fig.3 and in fig.1 (control 50S proteins) shows that the former contain spots at positions corresponding to those occupied by all normal 50S proteins. Examination of the stained gel slabs from which the drawings in figs.1 and 3 were made does however reveal that proteins which have been subjected to ammonolysis produce more diffuse spots than control proteins. This effect can also be seen in the upper panels of fig.3 in which the spots corresponding to cleavage products are abnormally elongated in the direction of first dimension electrophoresis. Analysis of the ammonolysis products obtained from 19i, 19S, j, and Ha shows that 19i and 19S have the same composition as complex 19 (L10, L11) and j and Ha the same composition as aK (L17, L32).

3.3. *Quantitative compositions of cross-linked complexes*

The quantitative composition of complexes can be beduced from two sets of data: (a) their observed mol. wts and qualitative compositions and the known mol. wts of ribosomal proteins; (b) the amounts of ^{35}S found in the cleavage products isolated after ammonolysis of complexes and the estimated sulfur contents of ribosomal proteins.

Data of these two kinds relevant to the cross-linked complexes discussed here and the quantitative compositions deduced from it are presented in the table.

4. Discussion

Proximity of proteins L10 and L11 in *E. coli* 50S ribosomes, confirmed by the results described here, has previously been indicated by the finding of these two proteins in three groups of complexes isolated from DMS treated ribosomes [9,10] and by studies of the reassociation of proteins L7/L12, L10 and L11 to 50S cores from which they have been removed [13].

As well as demonstrating that the peptide chains of proteins L2, L9; L10, L11 and L17, L32 approach each other closely and extensively enough in the intact 50S ribosomes to permit the formation of intermolecular cross-links by reaction of DMS with free NH_2 groups, the results described here show that the same protein pair can yield several separable cross-linked complexes. For example three complexes containing L10 and L11 (19, 19S, 19i, fig.1) and three containing

Table 1
Quantitative composition of some DMS cross-linked 50S protein complexes

Complex	Estimated mol. wt	Proteins detected in ammonolysis products: identity	mol. wt	sulfur content (atoms per mole)	measured radioactiv. total cpm ^{35}S (above background)	cpm ^{35}S per sulfur atom	Quantitative composition (mole ratio of identified cleavage products): mol. wt date	^{35}S measurement and content
3a	52 000–54 000	L2	28 800	9	3000	330	1:1	1:1
		L9	17 300	2	550	275		
19	39 000–				1730	250–290	1:1	1:1
					1550	220		
19S	41 000 +	L10	19 000	6–7	270	39–45	1:1	1:1
					210	30		
19i	37 000–38 000	L11	19 000	7	2100	300–350	1:1	1:1
					2200	310		
j	24 000–	L17	16 700	6	1350	225	1:1	1:1
					220	220		
Ha	26 000	L32	10 500	1	5000	830	1:1	1:1
					1000	1000		
aK					6000	1000	1:1	1:2
					2100	2100		

Data in columns 2 and 3 are derived from results presented in figs. 2 and 3, mol. wts of proteins L2, L9, L10, L11 and L17 are published values [14]. Sulfur contents of proteins L2, L10, L11 and L17 are based on published mol. wts [14] and amino-acid compositions [15,16]; Those given for proteins L9 and L32 were estimated as follows. 50S proteins extracted from *E. coli* MRE 600 labelled with ^{35}S during 6–8 generation times were separated by two-dimensional acrylamide gel electrophoresis. Spots corresponding to all 50S proteins were cut out of the dried gel slab by comparison with an autoradiograph and their ^{35}S content measured. The radioactivity per S atom was then calculated for proteins L10, L11, and L29 which are known to be unitary proteins in *E. coli* B [17] and which we assume to be unitary in *E. coli* MRE 600 also. A constant value was found and was used to calculate the number of sulfur atoms per molecule of proteins L9 and L32. (These calculations assume that *E. coli* MRE 600 50S ribosomes contain one copy per particle of proteins L9 and L32). Radioactivity measurements listed in columns 6 and 7 were made as follows. Regions centred on the positions of proteins L2, L9, L10 and L11 and corresponding in size to darkened areas in autoradiographs were cut out of dried gel slabs, as were regions covering stained areas corresponding to proteins L17 and L32, Gel samples were treated with 0.2 ml of 50% H_2O_2 at 50°C overnight in sealed vials, and radioactivity was then measured after addition of 1.5 ml of toluene–Triton X100 scintillation fluid.

L17 and L32 (j, Ha, aK; fig.1) migrate to neighbouring but different positions in two dimensional acrylamide gels under standard electrophoretic conditions (presence of urea). In addition each of the three L10–L11 complexes is resolved into two components by electrophoresis in the presence of SDS (fig.2A). The existence of multiple forms of the same complex could be due to the introduction of different numbers of cross-links between two proteins to the formation of cross-links at different sites, or to the presence of a single intermolecular cross-link at the same site together with a variable number of intramolecular crosslinks or of DMS residues which have reacted with a protein NH_2 group and a solvent molecule. The third possibility seems the most likely since the formation of cross-links between two ribosomal proteins at one or more of several different sites would require extensive proximity of their peptide chains. In addition we have observed in other experiments the conversion of protein S4 to an electrophoretically distinct form (spot N, fig.1b, [11], contains only S4) during treatment of E. coli 30S ribosomes with DMS.

Determination of the quantitative composition of complexes 3a, 19, 19S and 19i, is unambiguous since

proteins L2, L9 and L10, L11 are well resolved from each other and from other 50S species by two-dimensional gel electrophoresis. However, in the case of complexes j, Ha and aK ambiguity arises because proteins L17, L18, L19 (6,1, and 1–2 sulfur atoms respectively) and proteins L32 and L33 (1 sulfur atom each) are not well resolved in two-dimensional gels. Low resolution of these proteins is accentuated after reaction with DMS because as already mentioned protein samples which have been treated in this way produce more diffuse spots in two-dimensional gels than control proteins.

Careful comparison of the positions of stained spots in the dried gel slabs upon which the cleavage products of these complexes were separated and of spots in the autoradiographs of these gels shows that at least in the case of complexes j and Ha darkening of the X-ray films is centred on proteins L17 and L32. The amounts of ^{35}S in the cleavage products of complexes j and Ha shown in the table were measured in eluates of regions of dried gel slabs covering only the stained areas corresponding to proteins L17 and L32. When the total radioactivities in the larger gel regions corresponding to the darkened areas in the X-ray films were measured a stoichiometry of 1:1 (L17:L32) was again obtained. Analyses of the ^{35}S distribution in the cleavage products of complex aK show that it contains two moles of L32 per mole of L17 and we therefore consider it likely that this complex contains two or possibly three products i.e. L17–L32 and either or both of L18–L32 and L19–L32. The possibility that aK is a mixture of these three binary complexes is compatible with the similar mol. wts of L17, L18 and L19 (16 700, 14 300 and 14 900 respectively [14]).

Acknowledgements

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