PROTEIN NEIGHBORHOODS IN THE 30S RIBOSOMAL SUBUNIT OF *ESCHERICHIA COLI*

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1. Introduction

We have previously described the synthesis of a new series of cross-linking reagents which react through their azide-activated carbonyl groups with the aminogroups of proteins [1]. The particular virtue of these reagents is that they are derivatives of tartaric acid, which means that they can be cleaved by mild periodate treatment. The cleavability of protein complexes formed by such bifunctional reagents can be exploited in conjunction with special electrophoretic gel systems to simplify the analysis of the protein neighborhoods in ribosomes as well as other complicated macromolecular systems [1]. We now describe the identification of nine near-neighboring pairs of proteins in the 30S ribosomal subunit of *Escherichia coli* using these techniques.

2. Materials and methods

The crosslinking reagents tartryl diazide (TDA), tartryl di (glycylazide) (TDGA), and tartryl di-(ε-aminocaproyl azide) (TDCA) were prepared as described in [1]. Experiments were carried out with 30S ribosomal subunits prepared from Escherichia coli MRE 600 as described in detail earlier [2]. 30S subunits prepared from *Escherichia coli* PL1, a K strain, were used for the data in fig.1. Sodium metaperiodate (Merck) and periodic acid (Merck) were used interchangeably in the cleavage reaction. Additional experimental details are found in [1].

3. Results

When 30S ribosomal subunits are treated with TDA, TDGA, or TDCA, near-neighboring proteins are crosslinked together. The extent and variety of this crosslinking is apparent from the symmetrical two dimensional electropherograms illustrated in fig.1. In these gels the protein from the diazide-treated 30S subunits is fractionated on a first dimension, SDS-containing polyacrylamide gel. This gel is then soaked in a periodate solution, which results in the cleavage of the carbon-carbon bond between the vicinal hydroxyl groups of the crosslinking reagent and thereby releases member proteins of cross-linked complexes. The treated gel is then placed horizontally upon a slab gel of identical composition and electrophoresed in the second dimension. Uncleaved proteins migrate with the same mobility as in the first dimension, and will consequently lie on a diagonal. However, the cleavage products of the crosslinked complexes that were released by periodate treatment will exhibit a higher electrophoretic mobility in the second dimension than in the first. They are therefore found below the diagonal formed by the uncleaved proteins. The constituents of any cross-linked complex will lie directly below the electrophoretic position of that complex in the first dimension. Thus, this electrophoretic technique gives an immediate impression of the extent and variety of crosslinks produced by the various reagents.

From fig.1 it can be seen that there is an increasing amount and variety of crosslinked complexes formed with increasing length of reagent used. Furthermore, tentative identifications can be made for some of the

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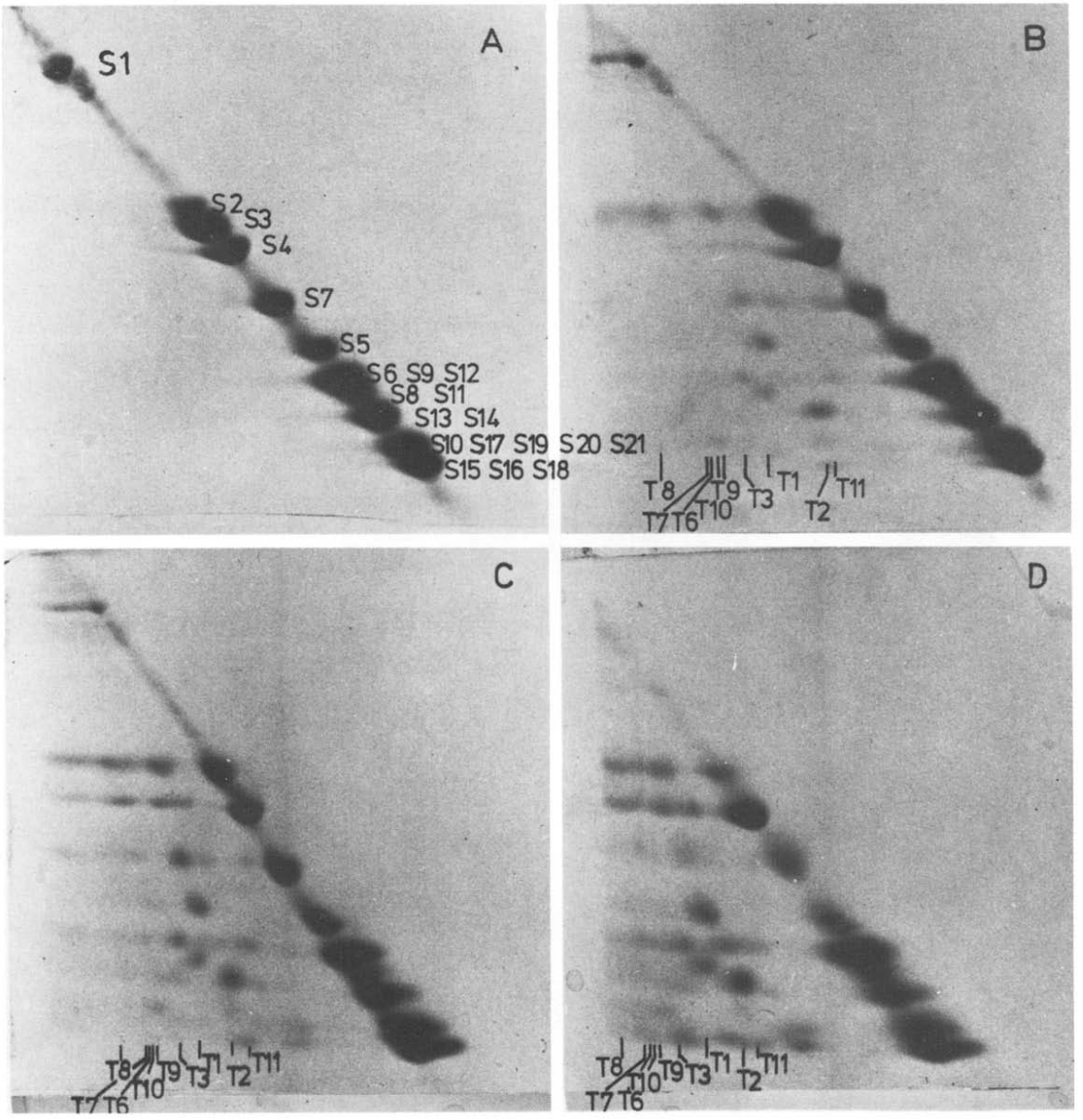


Fig.1. Symmetrical SDS-containing polyacrylamide electrophoresis of 30S total proteins. Samples were proteins from PL1 30S subunits which had been treated in the following manner: A) untreated control. B) 5 mM TDA. C) 5 mM TDGA. D) 5 mM TDCA. Details of the crosslinking reaction have been described previously [1]. Electrophoresis was performed essentially as described [1] except that the first dimension gel was shaken in two one-hour changes of PS buffer (20 mM NaH_2PO_4, 0.1% SDS, pH 6.0) containing 10 mM $NaIO_4$. The electrophoretic positions of the various complexes in the first dimension are indicated at the bottom of each figure. In order to better visualize T3 (a complex of S7--S9), PL1 (K strain) 30S subunits were used here instead of MRE 600 (C strain) 30S subunits because S7 from K strains has a higher molecular weight than S7 from C strains, and it accordingly separates well from T1 in material from a K strain. T3 is produced in similar yields in both strains.

complexes based on the fact that certain proteins have previously been found in the ribosome to be within crosslinkable distance from each other using diimidoesters. Thus complexes of S5–S8, [3], S7–S9 [4], and S13–S19 [4] can be located immediately. However, this electrophoretic technique can not be used alone for the identification of near neighboring proteins in the ribosome. Thus, electrophoresis in the present gel system does not adequately separate all of the crosslinked complexes, nor does it resolve all twentyone 30S proteins.

Rather than depend on a single electrophoretic step to separate the various crosslinked complexes from one another, we have fractionated the crosslinked protein by cellulose phosphate and Sephadex G100 chromatography as well as by preparative electrophoresis in SDS-containing polyacrylamide gels [2,4]. Each complex which we have isolated and subsequently identified has eluted from cellulose phosphate at an elution volume at or between those of its member proteins. Fig.2 shows one dimensional polyacrylamide gel electropherograms of purified T6, T7, and T9 along with their periodate-cleavage products. It can be seen from these electropherograms that the periodate cleavage is essentially quantitative for some complexes (T6, T7). Other complexes (T9 for example) are apparently cleaved to the level of only 80–90%, but this remaining material may in fact be non-crosslinked material which has cofractionated with the complex. In some cases the electrophoretic mobility of the cleavage products in SDS-containing polyacrylamide gel electrophoresis is slightly decreased.

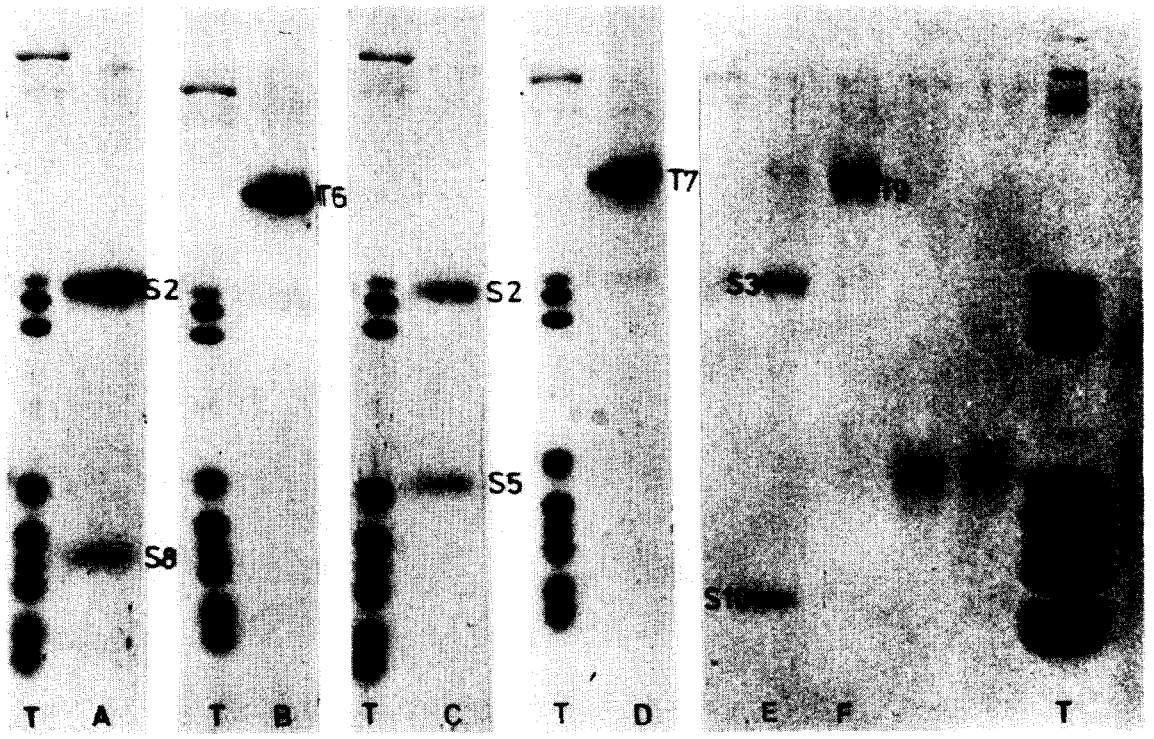


Fig.2. One-dimensional SDS-containing polyacrylamide gel electrophoresis of crosslinked complexes. Crosslinked complexes were isolated from TDA-treated 30S by cellulose phosphate and Sephadex G-100 chromatography as well as by preparative polyacrylamide gel electrophoresis [2–4]. One dimensional SDS-containing 15% discontinuous polyacrylamide gel electrophoresis was performed as described [11,12] in gel slabs. Periodate-cleavage in standard area buffer (6 M urea, 50 mM NaH_2PO_4, 12 mM methylamine, 0.7 mM 2-mercaptoethanol, pH 5.8) was accomplished by incubating the sample with 5 mM $NaIO_4$ for 30 min at room temperature in the dark (see [1]). A) Periodate-treated T6. B) Untreated T6. C) Periodate-treated T7. D) Untreated T7. E) Periodate-treated T9. F) Untreated T9. T) MRE 600 30S total protein.

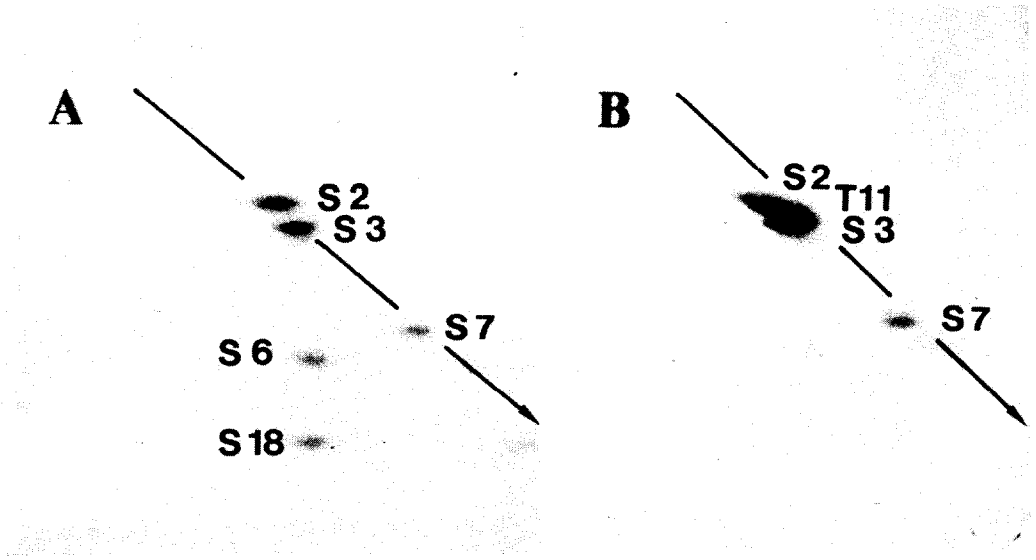
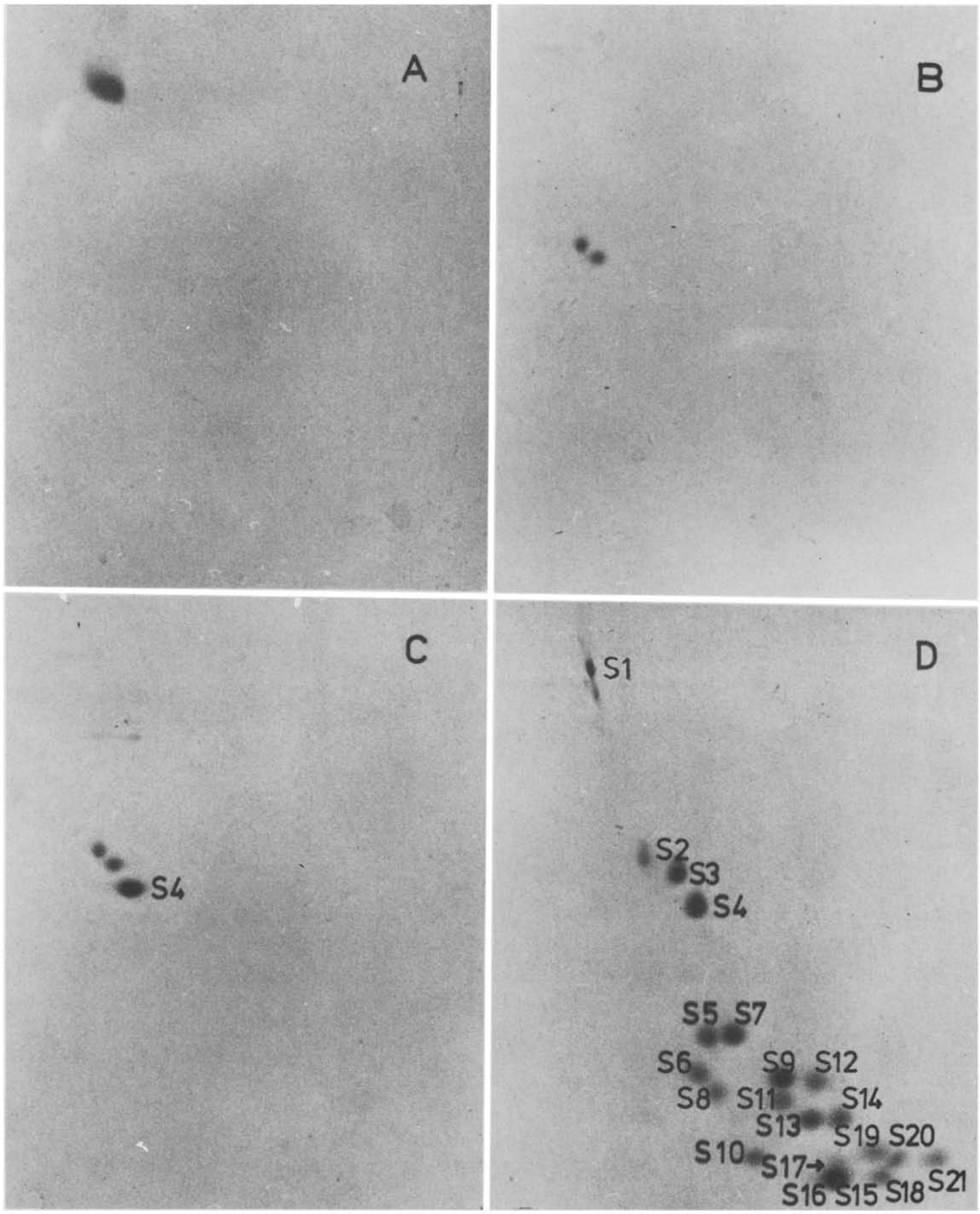


Fig.3. Symmetrical SDS-containing polyacrylamide gel electrophoresis of partially purified T11. A partially purified sample containing T11 was subjected to symmetrical electrophoresis as described in fig.1. Following the first dimension electrophoresis the gel was soaked in: A) PS buffer plus 10 mM $NaIO_4$. B) PS buffer only.

An example of this is apparent in fig.2C, where the S3 cleavage product has a slightly lower mobility than the S3 in the total 30S pattern.

Samples containing complexes which have not been purified so extensively as those in fig.2 can be analyzed using the symmetrical gel technique, thus simplifying considerably the identification process. Fig.3 shows a fractionated sample containing T11 which has been subjected to symmetrical gel electrophoresis. It can be seen that even though the T11 sample is composed of less than 50% crosslinked complex, the cleavage products (S6 and S18) can be distinguished from the cofractionating monomers (S3, S4 and S7). The unambiguous identification of the constituents for a purified crosslinked complex has been accomplished by the use of a modified two-dimensional electrophoresis system which separates nearly all of the 30S ribosomal proteins [1]. Fig.4 shows the identification of the components of T8. We have added purified protein S4 in order to locate the relative position of the two cleavage products in the total pattern. As can be seen by the comparison of fig.4c with fig.4d, the constituents of T8 are S2 and S3.

We have previously used immunological methods to identify the member proteins of complexes crosslinked with non-cleavable reagents [3–5], and we have used them here as a confirmatory identification procedure. All complexes were tested by Ouchterlony double diffusion using antisera raised against 20 of 21 purified 30S ribosomal protein (anti-S17 was unavailable). In fig.5 it can be seen that fusing precipitation lines are formed when a crosslinked complex is tested with antisera raised against the member proteins of the complex, indicating that the two antigens are part of the same complex. However, when the crosslink between the two proteins is cleaved by periodate, the precipitation lines cross, indicating that the two antigens are no longer part of the same complex. Thus, the availability of the cleavable crosslinker makes it possible to use Ouchterlony double diffusion to unambiguously identify the constituents of complexes even when the sample contains monomer contaminants. The identification systems described above have been applied to each of the following diazide-produced complexes: T1=S5–S8, T2=S13–S19, T3=S7–S9, T6=S2–S8, T7=S2–S5, T8=S2–S3, T9=S3–S10, T10=S4–S5, T11=S6–S18. The characterization of all of the near-neighbors listed were performed on complexes purified from TDA-crosslinked material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S16 S15 S18 S21

Fig. 4

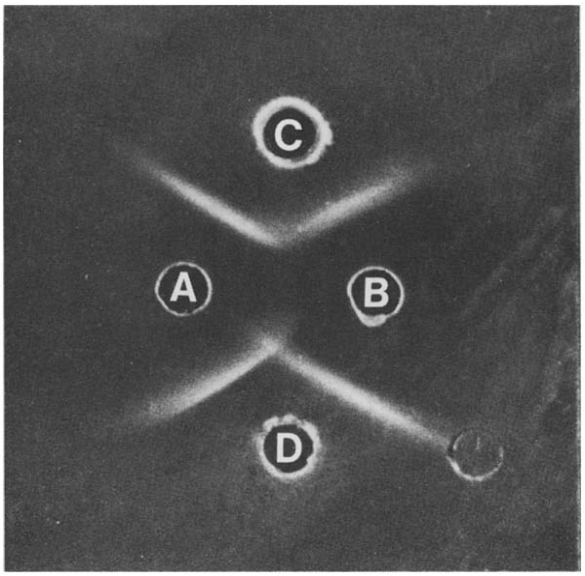

Fig.5. Ouchterlony double diffusion test to T10. Ouchterlony double diffusion tests were performed as described in [3]. The wells contained: A) Antiserum raised against purified protein S4. B) Antiserum raised against purified protein S5. C) Purified crosslinked complex T10 in standard urea buffer. D) The same sample as in C) which has been treated with 5 mM $NaIO_4$ for 30 min and stopped with a ten-fold molar excess of sucrose.

with the exception of S4–S5, which was purified in significantly higher yields from TDGA-crosslinked material. The relative yields of the cross-linked complexes varies considerably, as can be seen from fig.1. S5–S8, S7–S9, S13–S19, S3–S10, S2–S3, S6–S18 and S4–S5 are all apparent in the symmetrical electropherogram, whereas S2–S8 and S2–S5 are visible only after further fractionation of the total protein.

Finally, the molecular weights of each of the above named complexes as estimated from their mobilities on SDS gels agrees with the sums of molecular weight of their member proteins.

4. Discussion

Identification of the member proteins in cross-linked complexes has been the most troublesome part of recent attempts to explore ribosome topography with the aid of bifunctional reagents. The use of cleavable reagents such as the tartryl derivatives described in [1] greatly simplifies this work. However, the interpretation of neighborhoods established by these techniques requires further comment. The pairs of near-neighboring proteins S5–S8, S7–S9, and S13–S19, have been detected earlier with the aid of diimidoesters [3,4] and along with S18–S21 [3] as well as S14–S19 [5] are corroborated as near-neighbors in the functional ribosome on the basis of their correlation with other data such as the cooperativity expressed between the same proteins during ribosome assembly in vitro [2–8]. The new neighborhoods S2–S3, S2–S5, S2–S8, S3–S10 and S6–S18 are also predictable from the same sort of data. Mutations, expressed in either S4 or S5 are able to suppress the streptomycin dependent phenotype determined by alterations of S12 [9,10]. If it is assumed that such functional interactions reflect local interactions between these three proteins, then the neighborhood S4–S5 is also a predictable one. A more detailed discussion of the functional significances of the cross-linked pairs of proteins described here is found in [7].

The physical interpretation of the crosslink data is still more complicated. Although it might be tempting to conclude that proteins crosslinked by a 6 Å reagent such as TDA must have lysines at least within 6 Å of each other, this conclusion is dependent on the tacit assumption that the 30S subunit has a rigid

Fig.4. Two-dimensional electrophoresis of T8. Crosslinked complex T8 was prepared as described in fig.2. Since this sample had been exposed to SDS during purification, it was dialyzed against standard urea buffer (see fig.2). Then one-tenth volume of a settled slurry of Dowex 1X-2 (200–400 mesh, BioRad) was added and the sample was centrifuged [13]. The supernatant containing the SDS-free protein was then subjected to electrophoresis in a pH 4.5 urea-containing polyacrylamide gel followed by a second dimension electrophoresis in a SDS-containing discontinuous 15% polyacrylamide gel slab [11,12]. The samples were: A) T8 fraction. B) T8 which has been incubated with 10 mM $NaIO_4$ for 30 min. in the dark (see [1]. C) As in B) plus purified protein S4. D) MRE 600 30S total protein.

structure. There is no evidence for this assumption, and it is, therefore, possible, if not likely, that a flexible structure for the 30S subunit would permit some proteins to transiently approach within 6 Å of each other, even if their average separation were longer.

On the other extreme, the failure of a 24 Å reagent such as TDCA to give quantitative crosslinking of all the proteins does not necessarily mean that the proteins are in general so far apart that their lysines are separated by more than 20 Å. It is possible that steric hindrance from RNA intercalated between the proteins limits the crosslinking of proteins that are separated by distances that would otherwise be bridgeable by the present reagents. The low yield of crosslinked complexes obtained with the present reagents compared with what would be expected from oligomeric protein complexes is reminiscent of the results we obtained with the diimidoesters [3,4]. Here too we observe an increase in the yield of crosslinked complexes with increasing length of reagent (see fig.1). This length-dependence is more dramatic than that observed with the diimidoesters because it covers a larger size range (6 Å to 24 Å vs. 4.5 Å to 11 Å). Such data reinforce our earlier conclusion that the 30S proteins are not packed in RNA free domains, and are, therefore, likely to be dispersed in some fashion through a matrix of RNA.

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