THE USE OF A NEW SERIES OF CLEAVABLE PROTEIN-CROSSLINKERS ON THE *ESCHERICHIA COLI* RIBOSOME

L. C. LUTTER
Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

F. ORTANDERL and H. FASOLD
Institut für Biochemie, Johann Wolfgang Goethe-Universität, 6 Frankfurt/M., Sandhofstrasse, West Germany

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1. Introduction

Protein–protein crosslinking reagents have been used extensively to study the protein arrangements in the *Escherichia coli* ribosome [1–9], but these studies have been hindered by the difficulty of identifying the constituents of the new crosslinked complexes formed. A crosslinking reagent which could be cleaved under mild conditions would greatly facilitate this identification step. Crosslinking reagents containing a disulfide bridge have been described [9,10], but these preclude the use of reducing agents during the isolation of crosslinked complexes and, therefore, could conceivably allow non-neighboring proteins to become linked together through disulfide interchange in solution. In the present study we describe a new series of chemical crosslinking reagents of varying bridge length which contain vicinal hydroxyl groups. The crosslinks formed by these reagents can be quantitatively cleaved by mild treatment with periodate. Two polyacrylamide gel electrophoresis systems are also described which facilitate analysis of the complexes formed.

2. Materials and methods

2.1. *Tartryl-dihydrazide (TDH)**

20 g of tartaric acid diethyl ester (Merck Co.) were dissolved in 200 ml of ethanol and the solution was heated to the boiling point. After addition of 40 ml of hydrazine hydrate (80%, Riedel de Haen, Co.), the mixture was heated under reflux for 30 min, then cooled in an ice bath. The white crystals were filtered with suction, and the substance was purified by reprecipitation from aqueous solution with ethanol. The yield was 95%, the melting point 183°C [11].

2.2. *Tartryl-diazide (TDA)*

Just prior to further reactions the hydrazide was converted to the azide. 2.1 mmoles of sodium nitrite, dissolved in 0.5 ml of water, were added to a cold solution of 1 mmole of the dihydrazide in 5 ml of 2 N hydrochloric acid. After 10 minutes at 0°C a few grains of urea were added to destroy any excess nitrous acid. The diazide formed a slightly turbid solution.

2.3. *Tartryl-di(glycylhydrazide) (TDGH)*

4.2 g of glycine ethylester hydrochloride were dissolved in a suspension of 6.5 g of sodium bicarbonate in 50 ml of water. The mixture was cooled in an ice bath before tartryl diazide, freshly prepared from 10 mmoles of the dihydrazide as described above, was added in portions with thorough stirring and cooling. After 1 hr at 0°C the pH value of 7–8 was checked, and the mixture was extracted several times with ethyl acetate. The dried solution was evaporated to

Abbreviations: TDH, tartryl dihydrazide; TDA, tartryl diazide; TDGH, tartryl di(glycylhydrazide); TDGA, tartryl di(glycylazide); TDCH, tartryl di(ε-aminocaproylhydrazide); TDCA, tartryl di(ε-aminocaproylazide); SDS, sodium dodecyl sulfate; Tris, *tris*-(hydroxymethyl) aminomethane.

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dryness under vacuum and the residual oil was dissolved in 15 ml of ethanol. Then 2 ml of hydrazine hydrate (100%) were added. After standing at room temperature for several hours and in an ice bath overnight, the solution was decanted from the thick yellow oil, which gave white crystals after several reprecipitations from water by addition of ethanol. Yield: 20%, m.p.: 176–178°C.

Analysis: $C_8-H_{16}-O_6-N_6$
Calculated: C: 32.9%; H: 5.48%; N: 28.78%.
Found: C: 32.96%; H: 5.23%; N: 28.66%.

2.4. *Tartryl-di(ε-aminocaproyl hydrazide) (TDCH)*

The synthesis followed the procedure given for the glycyl derivative, but the molar ratio of the tartryl dihydrazide to amino acid ester was 1:6 instead of 1:3. The yield was 25%, m.p. 164–166°C.

Analysis: $C_{16}-H_{16}-O_6-N_6$
Calculated: C: 47.55%; H: 7.92%; N: 20.82%.
Found: C: 47.27%; H: 7.55%; N: 20.65%.

2.5. *Ribosomes*

30S ribosomal subunits were prepared from *Escherichia coli* PL1, a K strain, as described in detail earlier [12].

2.6. *Crosslinking*

Since the azides are unstable over long periods of time, they are prepared from the more stable hydrazides immediately before use. A typical crosslinking reaction employing a final diazide concentration of 5 mM was carried out as follows: 0.110 mmoles $NaNO_2$ in 0.100 ml H_2O was added to a solution of 0.050 mmoles of the dihydrazide in 0.31 ml in HCl and the solution was incubated at 0°C with shaking for 5 min. Then 0.020 mmoles of urea in 4.0 ml of H_2O at 0°C were added. This solution of the diazide was adjusted to pH 5–6 with 5 N KOH and to this was added 0.5 ml of 0.5 M triethanolamine–HCl, pH 8.5, 0.050 M $MgCl_2$, 0.4 M KCl. 5 ml of 30S subunits (100 A_{260}/ml) in Buffer A (0.050 M triethanolamine–HCl, pH 8.5, 0.005 M $MgCl_2$, 0.100 M KCl) were added and the solution was warmed to room temperature (22°C). The mixture was incubated for 30 min at room temperature and the reaction stopped by the addition of 0.100 ml of 2 M methylamine–HCl, pH 7.7. The 30S subunits were then centrifuged for 3 hr at 65 000 rev/min (Beckman Type 65) and resuspended in Buffer A to give a solution of 300 A_{260}/ml. These 30S subunits were extracted by the acetic acid technique and the protein dialyzed against standard urea buffer (6 M urea, 50 mM NaH_2PO_4, 12 mM methylamine 0.7 mM 2-mercaptoethanol, pH 5.8) [12].

2.7. *Symmetrical two-dimensional SDS polyacrylamide gel electrophoresis*

Symmetrical two-dimensional SDS polyacrylamide gel electrophoresis was performed by first electrophoresing crosslinked protein in a 15% discontinuous SDS-containing polyacrylamide gel [5,8,13]. This gel was then soaked for 5 hr in 100 ml of Buffer B (0.020 M triethanolamine–HCl, pH 7.5, 0.1% SDS) to remove most of the Tris because Tris reacts with $NaIO_4$. The gel was then soaked for 5 hr in Buffer B plus 15 mM $NaIO_4$. A slab gel of identical composition to the first dimension gel was then constructed. The periodate-treated first dimension gel was placed directly onto the polymerized lower gel of the slab and polymerized into place with spacer gel. The electrophoresis was then performed exactly as in the first dimension. Proteins were stained with Coomassie Brilliant Blue. Similar gel systems have been developed independently in the laboratories of Dr D. Elson and Dr R. Traut (personal communications).

2.8. *Modified two-dimensional electrophoresis*

Modified two-dimensional electrophoresis consisted of electrophoresis in the first dimension on a 'hard' pH 4.5 urea-containing polyacrylamide gel [14]. The gel was then removed from the tube, soaked for 3 hr in a buffer containing 0.025 M Tris–HCl, pH 7.2, and 1% SDS, and placed on an SDS-containing slab gel just as mentioned above. A small amount of phenol red marker dye solution was layered over the gel and the proteins were electrophoresed at 100 V until the marker dye left the gel. Proteins were stained as above.

3. Results and discussion

The reagents are all diazide derivatives of tartaric acid and are shown in fig. 1 along with their abbreviations. It is well established that, under the conditions used, the azide-activated carbonyl groups react readily with amino groups to produce amide linkages [15].

Sucrose gradient sedimentation profiles of untreated

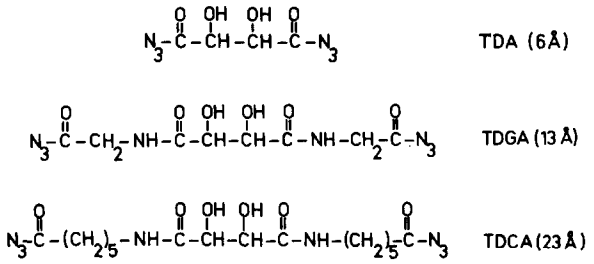


Fig. 1. Diazide tartarate derivatives. Numbers in parentheses indicate the distance between two protein amino groups which the reagent is capable of crosslinking.

and diazide treated 30S ribosomal subunits are indistinguishable, indicating no gross structural distortion has occurred. The TDA-treated 30S subunits are approximately 50% as active as the untreated subunits in poly-U dependent polyphenylalanine synthesis.

Initial experiments showed that when protein from diazide-crosslinked 30S subunits is electrophoresed in a one-dimensional SDS-containing polyacrylamide gel, several new high molecular weight components appear. When the crosslinked protein is treated with 5 mM periodate before electrophoresis, these new components disappear, indicating that the new components were crosslinked complexes which could be cleaved by periodate. However, disappearance of a new crosslinked component upon periodate oxidation does not necessarily mean that the complex has been cleaved to its monomer constituents. To demonstrate that this is in fact what has occurred, a symmetrical two-dimensional gel electrophoresis system has been employed. Protein from crosslinked 30S subunits is electrophoresed in a cylindrical, SDS-containing polyacrylamide gel. This gel is removed, incubated in a periodate solution to cleave the crosslinked complexes, and placed on a second dimension slab gel of identical composition to the first dimension. Uncleaved proteins will electrophorese with the same relative mobility as in the first dimension and will therefore lie on a diagonal. However, crosslinked complexes will be cleaved by the periodate treatment between dimensions and their components will exhibit a higher electrophoretic mobility in the second dimension than in the first. Consequently, they will be found below the diagonal that is generated by the uncleaved components.

The results of such a two-dimensional electrophoresis are shown in fig. 2. Untreated protein produces the expected diagonal pattern, while the cleavage products of many complexes are visible below the diagonal in the electropherograms of the crosslinked proteins. It is also apparent that the amount and variety of crosslinking is very dependent on the length of the reagent. TDA (6 Å) produces numerous crosslinked components. TDGA (13 Å) produces a slightly greater amount, and TDCA (23 Å) clearly produces the greatest amount and variety of crosslinking. This successively greater yield of crosslinked complexes with greater length of reagent is consistent with and extends previous crosslinking data found for the 30S subunit [1,6,8]. The continued increase of crosslinking using reagent lengths of greater than 10 Å is consistent with a model of the ribosome which has the protein and RNA more or less distributed throughout the particle as opposed to a model in which most of the protein is located in one portion of the ribosome and most of the RNA in another [16].

To facilitate unambiguous identification of the member proteins in a complex, a gel system is needed which can separate most if not all of the 30S proteins. The classical Kaltschmidt and Wittmann [17] system

Fig. 2. Symmetrical two-dimensional SDS gel electrophoresis. 30S subunits were treated with the various reagents as indicated and the extracted proteins were electrophoresed as described in Materials and methods. To the right is a one-dimensional electropherogram with the various bands identified. S3(5) and S3(9) are proteins 5 and 9, respectively, described in [12].

Fig. 3. Modified two-dimensional polyacrylamide gel electrophoresis. First dimension electrophoresis was from left to right in a 'hard' urea-containing pH 4.5 polyacrylamide gel. Second dimension electrophoresis was from top to bottom in a discontinuous SDS-containing polyacrylamide gel. Samples are: A.) 30S total protein B.) Protein from TDA-treated 30S subunits C.) The same sample as in B.) which had been treated with 5 mM $NaIO_4$ for 30 min at room temperature in the dark.

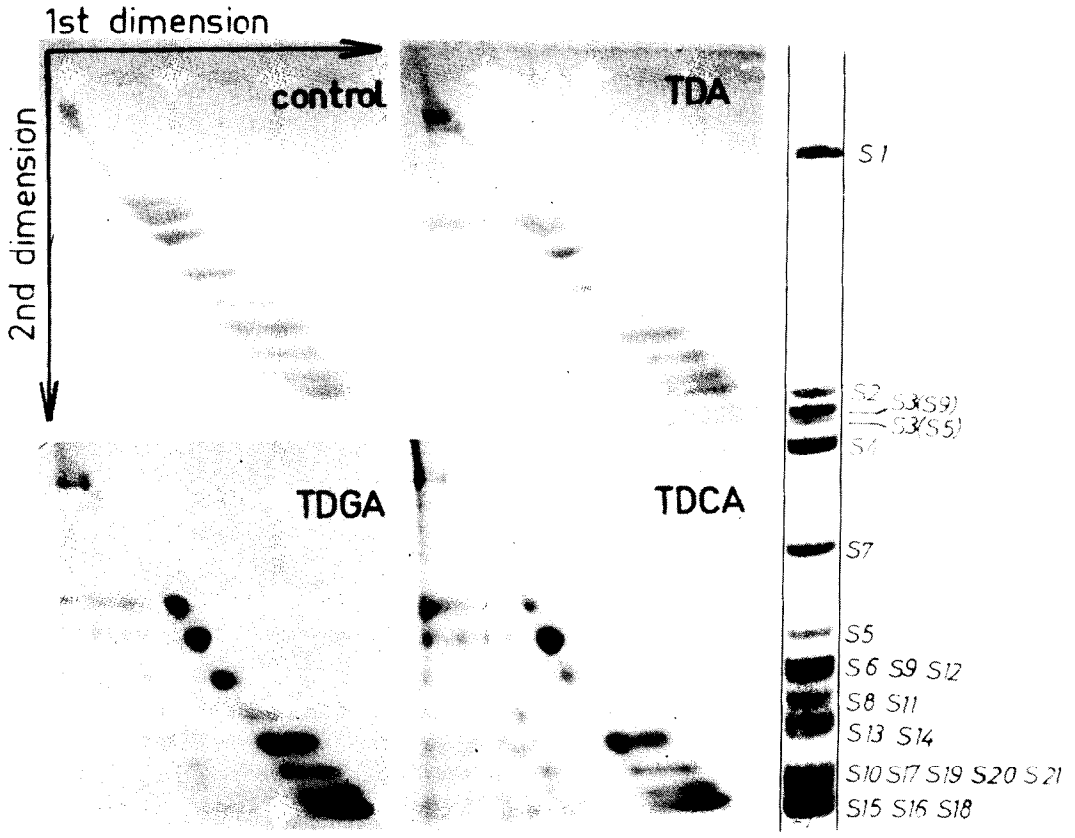
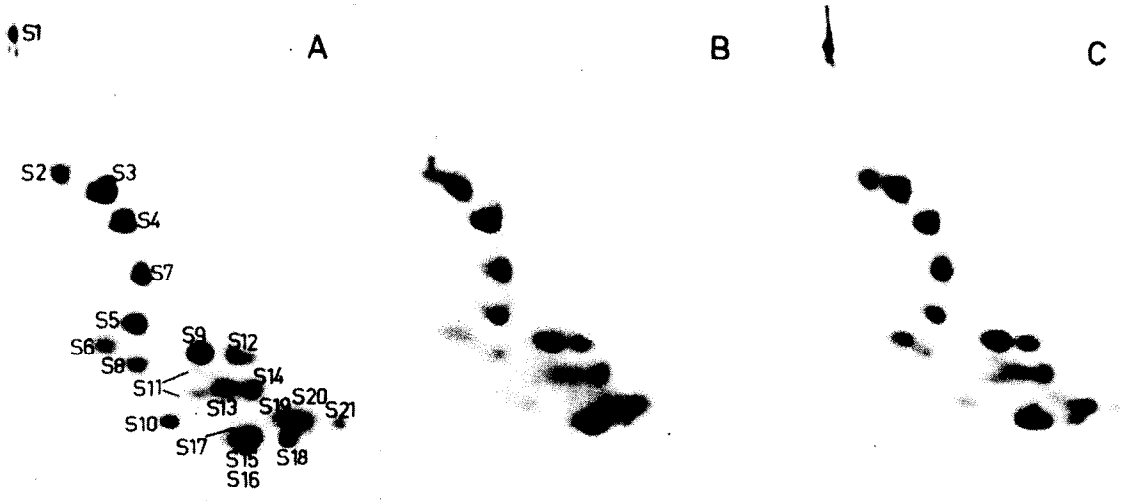

1st dimension
2nd dimension
control
TDA
TDGA
TDCA
S1
S2
S3(S9)
S3(S5)
S4
S7
S5
S6 S9 S12
S8 S11
S13 S14
S10 S17 S19 S20 S21
S15 S16 S18

Fig. 2


S1
A
B
C
S2
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S4
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Fig. 3

could be used, but since there is a net loss of one positive charge when a protein reacts with the azide, a gel system which minimizes the charge effect yet separates most of the proteins is desirable. Therefore we have used a polyacrylamide gel electrophoresis system consisting of a pH 4.5 urea-containing first dimension and an SDS-containing second dimension. The electropherograms in fig. 3 show that the cross-linked proteins show some smearing and double spot formation in the gel, but treatment with periodate results in the restoration of a pattern which can be used for identification purposes.

The approach which we are presently using to obtain protein neighbors involves crosslinking the 30S subunit with these reagents, separating the complexes by ion exchange chromatography or preparative gel electrophoresis, identifying the purified complexes by the modified two-dimensional technique described above and checking these identifications by Ouchterlony immunodiffusion tests using antisera raised against purified 30S proteins. The previously established neighborhoods of S5–S8 [4,5], S7–S9, and S13–S19 [8] have been confirmed using this approach. In addition, we have identified the protein neighborhoods S6–S18, S2–S8, S2–S5, S3–S10, S2–S3 and S4–S5 (manuscript in preparation).

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