Identification of a Cross-link in the *Escherichia coli* Ribosomal Protein Pair S13-S19 at the Amino Acid Level*

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*Escherichia coli* 30 S ribosomal subunits and 70 S ribosomes were treated with the bifunctional reagent diepoxybutane, acting as a cross-linker. One major cross-linked protein pair in the 30 S subunit was generated in relatively high yields. This cross-link was shown to consist of ribosomal proteins S13 and S19. Purification of this complex was achieved by a series of conventional and/or high pressure liquid chromatography techniques allowing its isolation in milligram quantities.

To reveal the exact position of the two amino acids involved in the cross-link formation, the purified protein pair S13-S19 was subjected to several enzymatic fragmentations, and the resulting peptides were characterized by sequence analysis, amino acid analysis, and fast atom bombardment mass spectrometry. After isolation of the cross-linked peptides, Cys* in protein S13 and His* in S19 could be unequivocally identified as the amino acids cross-linked by the bifunctional reagent. This result demonstrates that, despite neutron scattering data which place the centers of mass of S13 and S19 85 Å apart, at least these regions of the two proteins are located within a 4-Å distance in the ribosomal particle.

Therefore, in addition to electrophoretic techniques, other methods, such as using specific antibodies raised against the cross-linked ribosomal proteins, have been used for the identification of the cross-linked proteins (19). However, the most unambiguous way to identify the specific ribosomal proteins of the cross-linked pairs is to determine the characteristic amino acid sequences of the proteins or sequence regions, e.g. the N-terminal area. Since the complete amino acid sequences of all ribosomal proteins from *E. coli* have been elucidated (20), it is also possible to determine the cross-linked sites at the amino acid level, i.e. to identify the exact amino acid residue(s) in one protein cross-linked by the bifunctional reagent to the counterpart(s) in the other protein of the cross-linked protein pair.

In this paper, the large-scale isolation of the protein pair S13-S19, which has been cross-linked by the bifunctional reagent diepoxybutane in the 30 S subunit of the *E. coli* ribosome, is described. Furthermore, we present the identification of the specific cross-linking site at the amino acid level and discuss the topographical relationship of the proteins involved in this cross-link. These results are compared with data derived from other methodical approaches.

**Experimental Procedures**

**Results**

Proteins within the 30 S ribosomal subunit of *E. coli* were cross-linked with diepoxybutane. Identification of cross-linked protein pairs was done by one- and two-dimensional polyacrylamide gel electrophoretic techniques and by N-terminal sequence analysis. One cross-linked protein pair was obtained in high yields, the constituents of which were determined to be proteins S13 and S19 (14).

Preparative Isolation of Cross-Linked Proteins from 30 S Subunits—The purification of the cross-linked protein pair was accomplished by a series of chromatographic steps, starting with ion-exchange chromatography on conventional open columns and final purification by gel filtration HPLC (Fig. 1). This purification scheme was modified when the 70 S cross-linked ribosomal protein mixture was used instead of starting with cross-linked 30 S subunits. The use of 70 S ribosomal particles leads to a more complicated purification, but avoids the time-consuming and costly step of zonal centrifugation required for subunit preparation. In the case of

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Fig. 1. Purification scheme of cross-linked ribosomal protein pair S13-S19. Schematic presentation is shown of the purification procedure starting with *E. coli* cells and yielding the pure protein complex S13-S19. All major steps of the preparation scheme are shown. The procedure starting with 70 S total cross-linked protein differs from that with 30 S total cross-linked protein by one additional reversed-phase HPLC step due to the larger number of proteins. Details of the purification are given under "Experimental Procedures."

The 70 S cross-linked ribosomal protein mixture, an additional reversed-phase HPLC step after ion-exchange chromatography was introduced (Fig. 1). At first, a prefractionation step of the total protein extract from cross-linked ribosomes was performed by conventional ion-exchange chromatography on a CM-Sepharose CL-6B column (Fig. 2). The cross-linked proteins elute last under the conditions employed; thus, about 90% of the contaminating proteins can be removed in one purification step. Cross-linked proteins were detected by one- and two-dimensional polyacrylamide gel electrophoresis and by diagonal gel electrophoresis (see below). The cross-link-containing fractions were combined, desalted by dialysis, and subsequently lyophilized. As a second purification step, the proteins were separated on a Nucleosil C4 column (Fig. 3). The cross-link-containing fractions were again identified by two-dimensional and diagonal polyacrylamide gel electrophoresis prior to and after cleavage of the cross-linked protein pair (25, 26).

Finally, rechromatography was done on a gel filtration TSK C3000 SWP column, as shown in Fig. 4. The cross-linked ribosomal protein pair S13-S19 could be purified to homogeneity by this method. Identification of the protein constituents of the cross-linked protein pair was carried out by two-dimensional and diagonal micro polyacrylamide gel electrophoresis and N-terminal sequence analysis. Fig. 5a shows the untreated 30 S protein mixture used as reference, and Fig. 5b shows the total cross-linked 30 S protein mixture. Fig. 5(c and d) shows the purified cross-link prior to (c) and after (d) cleavage of the pair with sodium periodate. In Fig. 5d, the two constituents of the cross-link, namely proteins S13 and S19, are visible as isolated spots. N-terminal sequence analysis of a small amount (500 pmol) of the purified cross-linked protein pair confirmed the identity of the two proteins involved (data not shown).

Using the combination of the described methods, 5 g of 70 S starting protein (total protein extract) ultimately yielded approximately 4 mg (172 nmol) of purified cross-linked protein pair. At all steps of purification, the purity of the cross-link-containing fractions was checked by one-dimensional SDS gel electrophoresis according to Ref. 27 as shown in Fig. 6, starting from the 70 S cross-linked ribosomal protein mixture (Fig. 6, A-D) or, alternatively, from the 30 S cross-linked ribosomal protein mixture (Fig. 6, E-G).

Fragmentation of Cross-linked Protein Pair and Identification of Cross-linked Peptide Pairs—Staphylococcus aureus V8 protease proved to be the most useful one since it produced a
Amino Acid Cross-linking Site in Proteins S13 and S19

FIG. 2. Separation of 30 S cross-linked E. coli proteins. Prefractionation is shown of the 30 S cross-linked total protein mixture by conventional ion-exchange chromatography on a CM-Sepharose CL-6B column (5 × 30 cm) in equilibration buffer (10 mM phosphate buffer, pH 7.0, 6 M urea, 10 mM methylamine, and 6 mM β-mercaptoethanol) at ambient temperature. Lyophilized protein (1 g) was dissolved in 10–15 ml of this buffer and loaded onto the column. For elution, a linear gradient of potassium chloride (in the same buffer) from 0 to 0.5 M was applied. The flow rate was 4 ml/min, and 20-ml fractions were collected. The eluent was monitored at 280 nm. Small aliquots of each fraction were tested for the presence of cross-linked protein pairs by micro gel techniques and 12% polyacrylamide gel electrophoresis (see Fig. 6). The area where the cross-linked protein pair S13-S19 was marked by a peak containing the cross-linked protein pair S13-S19. The peak marked XL containing the cross-linked protein pair S13-S19 was collected on reversed-phase HPLC. The fractions obtained were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis (see Fig. 6) and two-dimensional polyacrylamide gel electrophoresis. The purity of the product was confirmed by gas-phase sequencing of a small aliquot.

FIG. 3. Reversed-phase HPLC of partially purified cross-linked protein fractions. Cross-linked protein fractions from preparative chromatography, shown in Fig. 2, were rechromatographed on a Nucleosil C4 column (250 × 16 mm) of 300-Å pore size and 5-μm particle diameter. The eluents were: buffer A, 0.1% aqueous trifluoroacetic acid in water; and buffer B, 0.1% trifluoroacetic acid in 2-propanol. The gradient applied was as follows: 25% buffer B for 10 min, 25–35% buffer B in 100 min, 35–80% buffer B in 5 min, and 80 to 25% buffer B in 5 min. The protein fraction XL of Fig. 2 (20–50 mg) was lyophilized, dissolved in 1–2 ml of buffer A, and injected. The eluent was monitored at 220 nm, 1.28 absorbance units at full scale. Recorder speed was 2 mm/min, flow rate was 3 ml/min, and fractions of 6 ml were collected. Aliquots of each fraction were tested for the presence of cross-linked protein pairs by two-dimensional and diagonal polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis (see Fig. 6). The area where the cross-linked complex S13-S19 elutes is marked XL.

small number of peptides, facilitating their characterization. Furthermore, digestion was carried out under acidic conditions (pH 4.1), which circumvented problems of solubility with the cross-linked proteins. The peptide mixture was separated on reversed-phase HPLC. The fractions obtained were directly analyzed by one-dimensional thin-layer chromatography (38), amino acid analysis, and manual and automated gas-phase sequence analyses. The localization of the cross-linking site is based on the known sequence of both proteins (S13 and S19) (39–41) which are involved in the cross-link formation.

With this information, the positions of the derived peptides in the proteins were determined by manual sequence analysis. Figs. 7 and 8 (upper) show the separation of the SP peptides...
and the marked positions of the peaks corresponding to the cross-linked peptide pairs obtained with different enzyme-substrate ratios. The peptides of these peaks were identified by rechromatography of each peak prior to and after sodium periodate cleavage (Fig. 8, lower). All peptides appeared at the same position, except for the cross-linked peptide pair which was cleaved by sodium periodate and yielded two new peaks in the HPLC trace eluting at different positions.

The cross-linked peptide pair produced by the second V8 digestion (SP2) consisted of two peptides with 36 (S13) and 8 (S19) amino acids, respectively, as determined by automated gas-phase sequence analysis. Fig. 9 shows the sequences of proteins S13 and S19 in comparison to the corresponding sequence of this peptide, i.e. S13 SP2-S19 SP2 (positions 82-117 in S13 and 65-72 in S19).

Since the peptides contained several amino acids which were possible candidates for involvement in cross-link formation, it was necessary to create smaller fragments by a second enzymatic digestion using thermolysin protease. Fig. 10 shows the separation of smaller sized peptides generated by digestion with thermolysin. The cross-linked peptide was identified in a manner similar to that used for the SP digests, using HPLC rechromatography prior to and after sodium periodate cleavage.

**Identification of Adjacent Amino Acids in Cross-linked Peptide Pair**—Characterization of the peptide pair generated by digestion with thermolysin was performed by automated gas-phase and solid-phase sequence analyses as well as by amino acid analysis. The purified peptide (1 nmol) was used for gas-phase sequence analysis. In Fig. 9, the particular part of the sequence covered by this peptide pair, i.e. S13 SP2Th-S19 SP2Th, is shown. The amino acid composition of the cross-linked peptide consisting of only 8 residues (positions 82-84 in S13 and 65-69 in S19) suggested three possible sites where cross-linking could have taken place, namely at the only possible amino acid residue of protein S13 (Cys24) and at 2 possible residues of protein S19 (His60 or Lys59). Sequencing indicated Cys24 (S13) and His60 (S19) as the 2 covalently attached residues since the following result was obtained (Table I).

**S13 Leu—Gly—X**
**S19 Met—Val—Gly—X—Lys**

No amino acid could be detected in Step 4 of the Edman degradation, whereas Lys59 was clearly identified in Step 5 as the authentic phenylthiohydantoin-lysine derivative. An additional differentiation between His60 and Lys59 of protein S19 as possible candidates for one of the cross-linking sites within the peptide pair was achieved by solid-phase sequencing. Approximately 500 pmol of the peptide pair were coupled...
w tative amino acid composition derived from the sequencing except for a gap obtained instead of CysM (S13) and HisGS peptide pair remained covalently attached to the glass after Again, LysG9 (S19) was positively identified in Step expected (released in a yield of about 30%). Amino acid data. Additionally, mass spectrometry of the peptide using expected (S13 SP2-Sl9 SP2). The material correspond-
tide could only couple to the diisothiocyanate glass by the N-
chain a-amino groups as detailed in Ref. 36. If the e-amino
new peaks corresponding to the two liberated peptides (S13 SP2 and
shorter gradient of 0-40% buffer in 120 min was applied. Only one peak elutes as expected (S13 SP2-S19 SP2). The material correspond-
to this peak was taken and rechromatography on reversed-phase HPLC. An aliquot of each peak (shown for peak XL (upper)) was taken and re injected under the same conditions as described for Fig. 7, except a shorter gradient of 0-40% buffer B in 120 min was applied. Only one peak elutes as expected (S13 SP2-S19 SP2). The material correspond-
ting to this peak was rechromatographed in a mixture of 10-15 mM sodium periodate for 10 min under identical conditions. The cross-linked peptide pair was then cleaved; hence, the peak disappeared, and two new peaks corresponding to the two liberated peptides (S13 SP2 and S19 SP2) appeared at earlier positions in the chromatogram.

to diisothiocyanate activated glass via N-terminal and/or side chain a-amino groups as detailed in Ref. 36. If the e-amino group of Lys was masked by cross-link formation, the pep tide could only couple to the diisothiocyanate glass by the N-terminal a-amino groups of the two peptides since S13 SP2Th contained no lysine residue (see Fig. 9). Then, as a consequence, the remaining peptide pair would be eluted from the glass support after the first cycle, rendering further Edman degradation of the peptide impossible. In fact, the cross-linked peptide pair remained covalently attached to the glass after the first step and could be sequenced to completion (Table 1), except for a gap obtained instead of Cys (S13) and His (S19); both residues could not be identified in the sequence. Again, Lys (S19) was positively identified in Step 5 as expected (released in a yield of about 30%). Amino acid analysis of the peptide confirmed the qualitative and quantitative amino acid composition derived from the sequencing data. Additionally, mass spectrometry of the peptide using the fast atom bombardment technique revealed a molecular ion peak at [M + H]+ = 948 exactly correlating to the mass of the cross-linked peptides plus a single connecting butane diol bridge (Fig. 11).

**DISCUSSION**

**Choice of Cross-linking Reagent**—In order to obtain meaningful cross-linking data, several requirements have to be fulfilled. First, the reaction has to be carried out under physiological buffer conditions; and the addition of organic solvents, which is often necessary to keep the cross-linking reagent in solution, should be avoided. Second, the reaction should lead to a relatively high yield (about 10%) of the cross-linked product under mild reaction conditions, and the product must be stable during all steps of the isolation procedure. Third, the bifunctional reagent should be cleavable since this leads to the individual proteins of the cross-linked protein pair which can be identified with ease and with less ambiguity than proteins within a cross-linked complex. Fourth, the charge of the bifunctional reagent should be neutral in order to avoid changes of the electrophoretic mobilities of the proteins caused by monovalent reaction of the reactive groups without formation of cross-links. Changes of the electrophoretic mobilities render the identification of the proteins by electrophoretic methods difficult and ambiguous.

We have tested a number of bifunctional reagents for their ability to generate cross-links of protein within the ribosomal particles (14) and have found that the 4-A cross-linker dipeoxybutane is most useful since this reagent fulfills all the requirements mentioned above. It predominantly reacts with nuclophilic centers at the amino acid side chains of lysine, cysteine, and histidine and, in case of the 30 S subunit of the E. coli ribosome, leads to one major cross-linked protein pair.  **Isolation of Cross-linked Protein Pair and Identification of Cross-linked Amino Acid Residues**—The results described in this paper were obtained by the application of advanced methods for the purification and characterization of ribosomal proteins and their peptides. These methods include various chromatography techniques and two-dimensional and diagonal polyacrylamide gel electrophoresis at a micro scale. As well, new procedures for desalting minute amounts of proteins and improved automated solid-phase and gas-phase protein sequencing have been crucial in these analyses. The application of these methods has not only led to the isolation of the cross-linked protein pair S13-S19 in a pure form, but has also enabled us to identify the precise amino acid residues (Cys in S13 and His in S19) of the pair S13-S19. This cross-link was made by treating E. coli 30 S subunits and 70 S ribosomes with dipeoxybutane in the ribosome under physiological conditions. The identification of the cross-link at the amino acid level has so far been performed only for two other pairs of ribosomal proteins (12, 13). In this context, two interesting points should be mentioned. First, the isolation of the protein pair S13-S19 and the identification of the cross-linked amino acids have been achieved without radioactive labeling. The use of a radioactive bifunctional reagent becomes complicated by the fact that most of the bifunctional molecules bind only monovalently to the protein, but do not lead to cross-linking; and the expected low amount of cross-linked protein moiety makes the identification of the cross-linked proteins or amino acids more difficult. Second, in order to obtain the cross-linked protein pair in an amount sufficient for the subsequent isolation of peptides for amino acid sequence analysis, we have started with 70 S ribosomes instead of 30 S ribosomal subunits in some experiments. In this way, the separation of the 30 S and 50 S
Amino Acid Cross-linking Site in Proteins S13 and S19

Fig. 9. Identification of cross-linked amino acids in ribosomal protein pair S13-S19. Presented are the two complete amino acid sequences of ribosomal proteins S13 and S19 and the sequences of the peptides obtained by enzymatic cleavage of the protein pair cross-linked by diepoxybutane. The peptide pair S13 SP1-S19 SP1 was produced by digestion of the cross-linked protein pair S13-S19 with S. aureus protease applying an enzyme:substrate ratio of 1:100, and the peptide pair S13 SP2-S19 SP2 applying an enzyme:substrate ratio of 1:10. The peptide pair S13 SP2Th-S19 SP2Th was derived by cleavage of the cross-linked peptide pair S13 SP2-S19 SP2 with thermolysin protease using an enzyme:substrate ratio of 1:50 (for conditions, see "Experimental Procedures"). Identification of the two cross-linked amino acids was done by sequence analysis of peptide pair S13 SP2Th-S19 SP2Th and confirmed by amino acid analysis and mass spectrometry (for details, "Results"). In the center of the figure, the cross-linked peptide pair S13 SP2Th-S19 SP2Th is shown with the bridging butanediol group.

Cross-linking of proteins within the ribosomal particles by short bifunctional reagents is an efficient way to reveal which proteins are close neighbors in situ and to check the results obtained by other methods. The identification of proteins S13 and S19 as neighbors within the 30S subunit of E. coli (1) is in excellent agreement with results from immune electron microscopy (16). However, from neutron scattering data (18), the distance between the centers of mass of proteins S13 and S19 is 85 Å. These two proteins, therefore, should not have been cross-linked, taking into account the short span (4 Å) of diepoxybutane and the small molecular weights (12,968 and 10,299, respectively) of the two proteins unless one or both of the proteins are elongated in the ribosome. The data, obtained on the smallest cross-linked peptide (S13 SP2Th-S19 SP2Th), by FAB mass spectrometry, clearly proved that only a spacer of four carbon atoms spans the distance between the cross-linked proteins S13 and S19.

It is interesting that the same proteins (S13 and S19) can be cross-linked by diepoxybutane not only in ribosomes from different E. coli strains (D10 and A19), but also in ribosomes from Bacillus stearothermophilus (44). This finding indicates that the topography of the ribosomes from Gram-positive B. stearothermophilus is very similar to that of Gram-negative E. coli, and it agrees with results from immune electron microscopy (16) which show that homologous proteins have the same locations in the ribosomes from these different bacterial species.

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subunits by zonal centrifugation, which is a time-consuming and yield-reducing step, could be avoided.

Topographical Aspects—A number of physical, chemical, and immunological methods are being used to elucidate the topography of the ribosomal particles, i.e. the spatial arrangement of the many ribosomal components in situ. Among them are immune electron microscopy (16, 17), neutron scattering (18), cross-linking of proteins and/or RNAs (19, 42) and, of course, crystallization of ribosomal particles followed by x-ray structure analysis (43). The results from these studies have already given considerable insight into the topography of ribosomes.
Amino Acid Cross-linking Site in Proteins S13 and S19

Fig. 10. Identification of cross-linked peptide pair derived from thermolysin cleavage of staphylococcal fragment S13 SP2-S19 SP2. Fragmentation is shown of the staphylococcal S13 SP2-S19 SP2 cross-linked peptide (see Fig. 9) by thermolysin. This peptide (10-30 nmol) was digested with thermolysin as described under "Experimental Procedures." Aliquots of the digest were separated on a LiChrosphere C8 column (column parameters and separation conditions were as described for Fig. 7, except for the gradient, which was applied as follows: 0-25% buffer B in 90 min, 25-80% buffer B in 5 min, and 80 to 0% buffer B in 5 min, followed by re-equilibration for 30 min). The peaks obtained were tested for the presence of a cross-linked peptide pair by the same techniques as described for Fig. 7. The peak marked XL was found to consist of two peptides of 3 residues (S13: Leu<sup>s</sup> to Cys<sup>s</sup>) and 5 residues (S19: Met<sup>6</sup> to Lys<sup>6</sup>), respectively. These peptides were identified by gas-phase and solid-phase sequence analyses, amino acid analysis, and FAB mass spectrometry.

Table I

<table>
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<td>Lys&lt;sup&gt;*&lt;/sup&gt;</td>
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| * Attached to the solid support; positively identified by gas-phase sequencing.

In conclusion, the techniques described in this paper, which now allow the identification of specific amino acids involved in protein-protein or protein-RNA cross-links, initiate a new, powerful approach to study the topography within ribosomes that will complement other physical and biochemical approaches. At present, our group is involved in work on the amino acid identification of several additional protein-protein cross-links in bacterial ribosomes which we hope will also shed light upon the higher order structures of the ribosome.

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REFERENCES


Fig. 11. FAB mass spectrum of cross-linked peptide pair S13 SP2Th-S19 SP2Th. For sample preparation, 100 pmol of peptide in 100 µl of 5% acetic acid and 10% glycerol were concentrated in a Speed Vac concentrator. 0.2 µl was subjected to FAB mass spectrometry (Vacuum Generator ZAB-3HF/BEB mass spectrometer; source temperature, 20 °C; pressure, 0.67 millipascal; accelerating voltage, 8 kV; FAB gun emission, 0.2 mA; mass resolution, 1500).


Amino Acid Cross-linking Site in Proteins S13 and S19


EXPERIMENTAL PROCEDURES


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250 μm column size) using a solvent system made of 0.1 N aqueous TFA and acetonitrile.

Digestion of a Staphylococcal aureus generated crosslinked peptide pair by thermolysin to obtain smaller fragments of the peptides derived from V8 protease cleavage of the crosslinked protein pair S13-S19. A second enzymatic digestion was performed using thermolysin in 0.1 M N-ethylmorpholine buffer at pH 8.1 for 2 h at 50°C. The amounts of peptide per experiment varied from 10 to 50 nmol. The enzyme to substrate ratio was 1:50.

Separation of thermolysin-generated peptides on reversed-phase HPLC. The small fragments of the crosslinked SP-peptide were separated on reversed phase HPLC using a LiChrospher CB column. Merck, Fiez) of 100 μm pore size, 5 μm particle size (4 x 250 μm column size) and a gradient made of 0.1 N aqueous TFA and acetonitrile. The fractions obtained were analyzed by thin layer chromatography, amino acid analysis, and automated sequence analysis.

Sequence analysis of crosslinked peptides and proteins—Manual sequence analysis of protein and peptide samples was performed by the DART/FTICR double-coupling technique according to (21, 39). Samples from 500 pmol to 5 nmol. derived from HPLC separations directly after drying of the fractions, were used for sequence analysis. This method was mainly employed for identification of the peptides after HPLC, since it allows the analysis of many samples at a time.

Automated gas phase sequencing was done on a gas phase sequencer (22) constructed in the MPI workshop and operated according to (24) and on the Applied Biosystems sequencer, Model 477A and analyzer Model 130A (Applied Biosystems, CA). Samples from 100 pmol to 1 nmol were sequenced by this method.

Automated solid phase sequencing was done on a modified LKB solid phase sequencer. Model 4020, according to the method described in (25). Attachment of peptides and proteins to the sequencing support was by lysine coupling to ETP-activated glass (26). Samples from 500 pmol to 5 nmol were sequenced.

Amino acid analysis of peptide—Hydrolysis of the peptides was performed in 100 μL 6.7 N HCl for 24 h at 110°C in vacuo. The amino acids were determined after pre-column derivatization with n-phthalaldehydethiohydantoin (NPHTH); by reversed phase HPLC, as described in (37).