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Cross-Linked Amino Acids in the Protein Pair S13-S19 and Sequence Analysis of Protein S13 of *Bacillus stearothermophilus* Ribosomes

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ABSTRACT: The 30S ribosomal subunits from *Bacillus stearothermophilus* were cross-linked under native conditions with the bifunctional reagent diepoxybutane. The dominant protein-protein cross-link in the 30S ribosomal subunit between proteins S13 and S19 [Brockmöller, J., & Kamp, R. M. (1986) *Biol. Chem. Hoppe-Seyler 367*, 925-935] was isolated on a preparative scale. The presence of a single cross-link site between cysteine-83 of protein S13 and histidine-68 of protein S19 was established by microsequence analysis of isolated cross-linked peptides. This cross-link site was further confirmed by different analytical methods including fast atom bombardment mass spectrometry of the cross-linked peptide. The cross-linking site is located in the highly conserved C-terminal regions of proteins S13 and S19. In addition, the complete amino acid sequence of protein S13 from *B. stearothermophilus* is determined. Sequence comparison with the homologus *Escherichia coli* protein S13 revealed 58% identical amino acid residues.

Protein cross-linking has found wide application in the study of the protein topography of prokaryotic and eukaryotic ribosomes. The results from this method combined with those from other approaches have already given valuable information about the topography of ribosomes (Hardesty et al., 1986). For construction of more detailed structural models of the ribosomal particles, further cross-linking studies are necessary for the exact determination of the cross-linking sites at the amino acid level. At this time in the cross-linked *Escherichia coli* ribosomal protein pair S5-S8 (Allen et al., 1979) and within a cross-linked pair of the proteins L7/L12 (Massen et al., 1981) have the two cross-linked amino acids been identified.

In recent years considerable progress in crystallizing ribosomes, ribosomal subunits, and ribosomal proteins has been achieved. Such crystals are mainly obtained from *Bacillus stearothermophilus* and from halobacterial ribosomes (Yonath et al., 1986). These studies are complemented by direct amino acid sequence analysis of ribosomal proteins from these organisms. Since the amino acid sequences of all ribosomal

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proteins from *E. coli* and most from *B. stearothermophilus* are known (Wittmann-Liebold, 1986b), the latter ribosomes were chosen for determination of cross-linking sites on the amino acid level, in order to provide additional and complementary structural information. By sequence comparison between homologous ribosomal proteins derived from *B. stearothermophilus* and *E. coli* a different degree of homology was found between both sets of proteins, which was highest for functionally important proteins. High homology between ribosomal proteins from these organisms was also shown by heterologous reconstitution experiments (Higo et al., 1973; Wrede et al., 1973) and by immunological cross-reactivities (Geisser et al., 1973; Isono et al., 1973; Fahnestock et al., 1981).

In the present paper we describe the identification of the cross-linked amino acids in the ribosomal protein pair S13–S19. This protein pair was obtained by cross-linking intact 30S ribosomal subunits with the bifunctional reagent diepoxybutane. This reagent has already been applied as a protein–RNA cross-linking reagent in the *E. coli* ribosome (Bäumert et al., 1978).

In an accompanying study the complete amino acid sequence of protein S19 of *B. stearothermophilus* has recently been determined (Hirano et al., 1987) and was found to have a surprisingly high homology (71%) to the corresponding *E. coli* protein (Yaguchi et al., 1978). In order to allow also the assignment of the cross-linking residue in the *B. stearothermophilus* proteins S13, whose sequence was not yet available, the complete amino acid sequence of protein BST S13 needed to be determined and is presented in this paper. Its sequence homology to protein S13 of *E. coli* is discussed.

Materials and Methods

Chemicals. DL-1,2:3,4-Diepoxybutane was purchased from Merck (Darmstadt, FRG). 1,4-Dibromo-2,3-butanedione was from Aldrich (Milwaukee, WI). [^{3}H]sodium borohydride and [^{14}C]iodoacetic acid were from Amersham (Braunschweig, FRG).

Staphylococcus protease was purchased from Miles (Naperville, IL), thermolysin (5× crystallized) was from Serva (Heidelberg, FRG), and trypsin and clostripain were from Cooper (New Jersey). Lysyl-endopeptidase was obtained from Wako-Pure Chemicals (Tokyo, Japan).

2-Propanol and acetonitrile were LiChrosolv grade from Merck. Solvents and chemicals for Edman degradations using the manual DABITC–PITC[1] double-coupling method were purified as described (Wittmann-Liebold et al., 1984) and for automatic gas-phase sequencing were purified according to Reimann et al. (1986).

Acrylamide and *N,N'*-methylenebis(acrylamide) were from Serva (twice crystallized). All other chemicals were of analytical grade and purchased from Merck.

HPLC supports were Vydac C_4 (5-μm particle size and 300-Å pore size) and Vydac C_{18} (10 μm, 300 Å) from The Separation Group (Hesperia, CA). TSK ODS 120T support was from Toyo Soda (Tokyo, Japan).

Preparation of Ribosomes. Growth of *B. stearothermophilus* (strain 799), preparation of ribosomes and ribosomal subunits, and ribosomal protein extraction by the acetic acid treatment (Hardy et al., 1969) or by a modified LiCl procedure have been described earlier (Brockmöller et al., 1986).

Synthesis of Tritiated Diepoxybutane. DL-1,2:3,4-Diepoxybutane (synthesis grade) was used without further purification. Tritium-labeled diepoxybutane was synthesized in collaboration with Professor H. Fasold, University of Frankfurt, by reductive tritiation of 12 mg (i.e., 50 μmol) of 1,4-dibromo-2,3-butanedione with 20 μmol (10 mCi) of [^{3}H]sodium borohydride. Epoxy groups were formed from the resulting dibromobutanediol by the addition of a total of 95 μmol of NaOH over 90 min as small aliquots.

Cross-Linking. The cross-linking reaction was performed in the following buffer: 20 mM triethanolamine hydrochloride, pH 7.9, 10 mM $MgCl_2$, 50 mM KCl, and 6 mM β-mercaptoethanol (TMK buffer). Ribosomes or ribosomal subunits were dialyzed against this buffer overnight before cross-linking and then preincubated at 37 °C in a water bath with gentle shaking for 15 min at a ribosome concentration of 100 A_{260} units/mL. An equal volume of prewarmed TMK buffer containing 2% freshly dissolved diepoxybutane was added to the ribosome solution. The cross-linking reaction was allowed to take place for 2 h and stopped by the addition of a twofold molar excess of 2 M methylamine hydrochloride (pH 7.9) solution over diepoxybutane (i.e., 30 mL of 2 M methylamine hydrochloride per 100 mL of ribosome solution). For cross-linking with [^{3}H]diepoxybutane, 500 A_{260} units of 30S subunits was incubated with 3 mCi of [^{3}H]diepoxybutane (i.e., about 50 μmol of diepoxybutane) in 10 mL of TMK buffer for 2 h, followed by 1-h incubation with an additional 50 μL of nonradioactive diepoxybutane.

Periodate Cleavage. Cross-linked proteins were cleaved for 10 min in 10 or 20 μL of 100 mM acetate buffer at pH 4 containing 10 mM freshly dissolved sodium metaperiodate. Oxidation was stopped by direct injection onto HPLC columns or by lyophilization and suspension in electrophoresis buffer containing 2-mercaptoethanol.

Polyacrylamide Gel Electrophoresis. One-dimensional SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970), and the two-dimensional gel electrophoresis was performed according to Geyl et al. (1981).

Isolation of Proteins. Isolation of the protein pair S13–S19 on a large scale has been described in detail (Brockmöller et al., 1986). Briefly, the cross-link was isolated by stepwise LiCl extraction and HPLC gel filtration. In some instances, the isolated cross-link was further repurified by reversed-phase chromatography on a Vydac C_{18} column.

The single proteins S13 and S19 were isolated by a one-step reversed-phase chromatography, as shown earlier (Brockmöller et al., 1986), on a Vydac C_4 support with a shallow gradient of aqueous 0.1% TFA as buffer A and 2-propanol containing 0.1% TFA as buffer B.

Protein S13 was also isolated in high purity by a one-step reversed-phase chromatography on a diphenyl column (J. C. Baker) in the same buffer system.

Separation of Peptides. Peptides were separated on a laboratory-packed TSK ODS 120 T column (4 × 250 mm) with a Beckman HPLC gradient system and aqueous 0.1% TFA as buffer A and 80% acetonitrile containing 0.1% TFA as buffer B. The column temperature was 35 °C, and the flow rate was 0.7 mL/min. The cyanogen bromide peptides were separated by gel filtration on a TSK 2000 SW column with 100 mM ammonium acetate buffer at pH 4.1 as eluent and a flow rate of 50 μL/min.

Chemical and Enzymatic Cleavages. Cyanogen bromide cleavage was performed according to Gross et al. (1961).

[1] Abbreviations: BST, *Bacillus stearothermophilus*; SP, *Staphylococcus aureus* (V8) protease; Cl, clostripain; K, lysyl-endopeptidase; HPLC, high-performance liquid chromatography; OPA, *o*-phthalaldehyde; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; DABITC, 4-(*N,N*-dimethylamino)azobenzene-4'-isothiocyanate; PITC, phenyl isothiocyanate.

Digestion with staphylococcus protease was performed in 100 mM ammonium acetate buffer at pH 4.1 for 48 h at 37 °C with an enzyme–substrate ratio of 1:15 and a second addition of enzyme (1:15) after 24 h. In most preparations the cross-link S13-S19 was digested immediately after separation on the gel filtration column directly in the gel filtration buffer. In this way, losses of protein material and solubility problems were avoided. All digestions were stopped by injection onto HPLC columns. Digestions with trypsin and lysyl-endopeptidase were performed in 100 mM *N*-methylmorpholine buffer, pH 8.2, at 37 °C for 4 hours with an enzyme–substrate ratio of 1:50. Digestion with thermolysin was performed at 50 °C in 100 mM *N*-methylmorpholine buffer, pH 8.2, for 2 h with an enzyme–sustrate ratio of 1:50. Digestion with clostripain was performed in 25 mM sodium phosphate buffer at pH 7.6 containing 2.5 mM dithioerythritol for 8 h with an enzyme–substrate ratio 1:50.

Amino Acid Analysis. Peptides were hydrolyzed for 20 h at 110 °C in 5.7 M HCl containing 0.02% β-mercaptoethanol and 0.001% phenol after evaporation and flushing with nitrogen. Amino acid composition of protein and peptide hydrolysates was determined by the automated OPA precolumn derivatization method (Ashman et al., 1985).

Edman Degradations. Manual degradations were done with an improved version of the DABITC–PITC double-coupling method (Wittmann-Liebold et al., 1986). Three larger peptides (SP9; K15; Cl13) and the intact protein S13 were sequenced in a gas-phase sequencer (Applied Biosystems, Model 477A). A total of 5 nmol of the N-terminal SP peptide of protein S13 (SP12) was coupled to 20 mg of aminopropyl glass via the carboxyl groups after carbodiimide activation (Wittmann-Liebold, 1986a). This peptide was sequenced in a modified (LKB, Model 4020) solid-phase sequenator by the DABITC–PITC double-coupling method (Salnikow et al., 1981). By the same attachment procedure, 1 nmol of the C-terminal peptide of protein S13 obtained from digestion with thermolysin was coupled to 3 mg of aminopropyl glass. This was sequenced in the gas-phase sequencer; the glass beads were kept between two glass fiber filters. A total of 3 nmol of cross-linked peptide obtained from SP and thermolysin digestion was coupled via the amino terminus and the ε-amino groups of the lysine to diisothiocyanate-activated aminopropyl glass and degraded in an LKB solid-phase sequenator.

Results

For determination of the cross-linking site, the isolated protein pair S13–S19 was cleaved by chemical and enzymatic methods. The peptides obtained were separated and analyzed for cross-linked peptides. At first, the larger sized cyanogen bromide and staphylococcal protease fragments were analyzed, and by recleavage of these fragments cross-linked peptides containing only a few amino acids were obtained. The use of tritiated diepoxybutane facilitated the identification of cross-linked peptides, but the proof of the cross-linking site was accomplished by a combination of sequence analysis, HPLC before and after cleavage of the cross-linking bond, and solid-phase sequencing and mass spectrometry.

Cross-Linking Experiments. The 30S ribosomal subunits and 70S ribosomes (tight couples) of *B. stearothermophilus* were treated with the bifunctional reagent diepoxybutane under native conditions. The ribosomal proteins were extracted by acidic acid treatment or LiCl extraction and were investigated by diagonal and two-dimensional polyacrylamide gel electrophoresis. Only one protein–protein cross-link was formed in high yields. This cross-link was formed irrespective of whether isolated 30S subunits or whole 70S ribosomes were used for cross-linking. The two protein constituents of this cross-link were identified as proteins S13 and S19 by N-terminal microsequencing and electrophoretic methods.

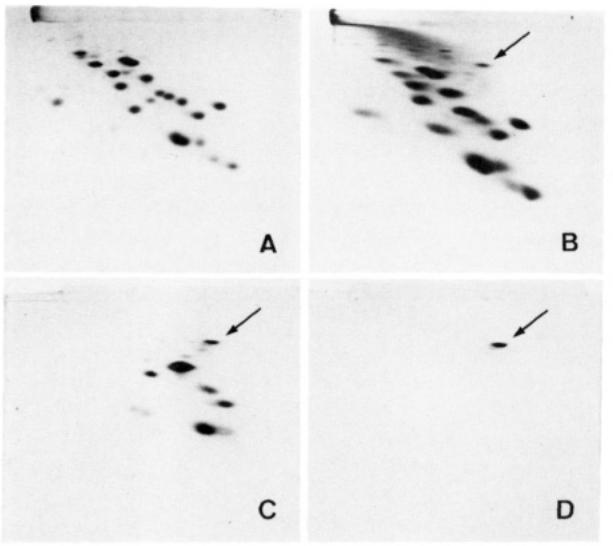

FIGURE 1: Isolation of the cross-linked protein pair S13–S19. The 2D electropherogram of the BST 30S acetic acid protein extract (A). (B) The 30S protein extract from diepoxybutane cross-linked 30S subunits. The LiCl-extracted protein fraction, enriched in the protein pair S13–S19, is shown in (C) and the isolated cross-link S13–S19 in (D). The arrow marks the position of the cross-linked protein pair.

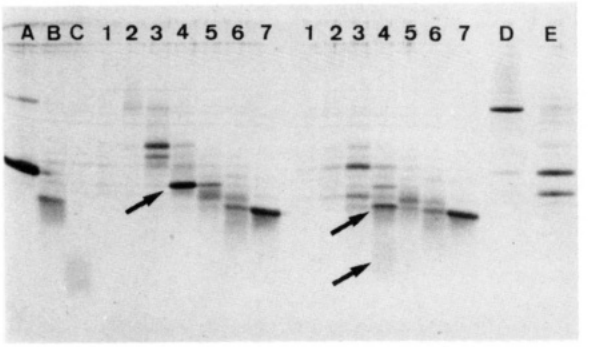

FIGURE 2: Isolation of cross-linked CNBr peptides derived from S13–S19. This SDS–polyacrylamide gel shows the fragments after fractionation by gel filtration HPLC prior to cleavage (fractions 1–7, right) and after periodate cleavage (fractions 1–7, left). The cross-linked peptide and the two peptides which appeared after cleavage of the cross-linked peptide are marked by arrows. Lanes A–C show molecular weight standards [bovine cytochrome *c* (12 300), porcine ACTH (4500), and bovine insulin B chain (3500)], land D shows the cross-link S13–S19, and lane E shows the proteins S13 and S19 resulting from periodate cleavage of the cross-link.

Some additional cross-links could be detected in the higher molecular weight area of the two-dimensional electropherograms, but they were formed in much lower yields and hence not further investigated. The yield of the pair S13–S19 varied between 5 and 10% as estimated through comparison of the intensity of the corresponding spots in the electropherograms (Figure 1). A total of 3 mg of pure cross-link was isolated from 150 000 A_{260} units of cross-linked 30S subunit and was subjected to the experiments at the peptide and amino acid level described below.

CNBr Fragmentation. The cross-linked protein pair S13–S19 was cleaved with cyanogen bromide. The fragments were separated by HPLC gel filtration on a TSK 2000 SW column, and the fractions were tested for the presence of cross-linked peptides by SDS–polyacrylamide gel electrophoresis prior to and after periodate cleavage of each fragment. [A cross-linked peptide pair is visible as a homogeneous band (Figure 2) before

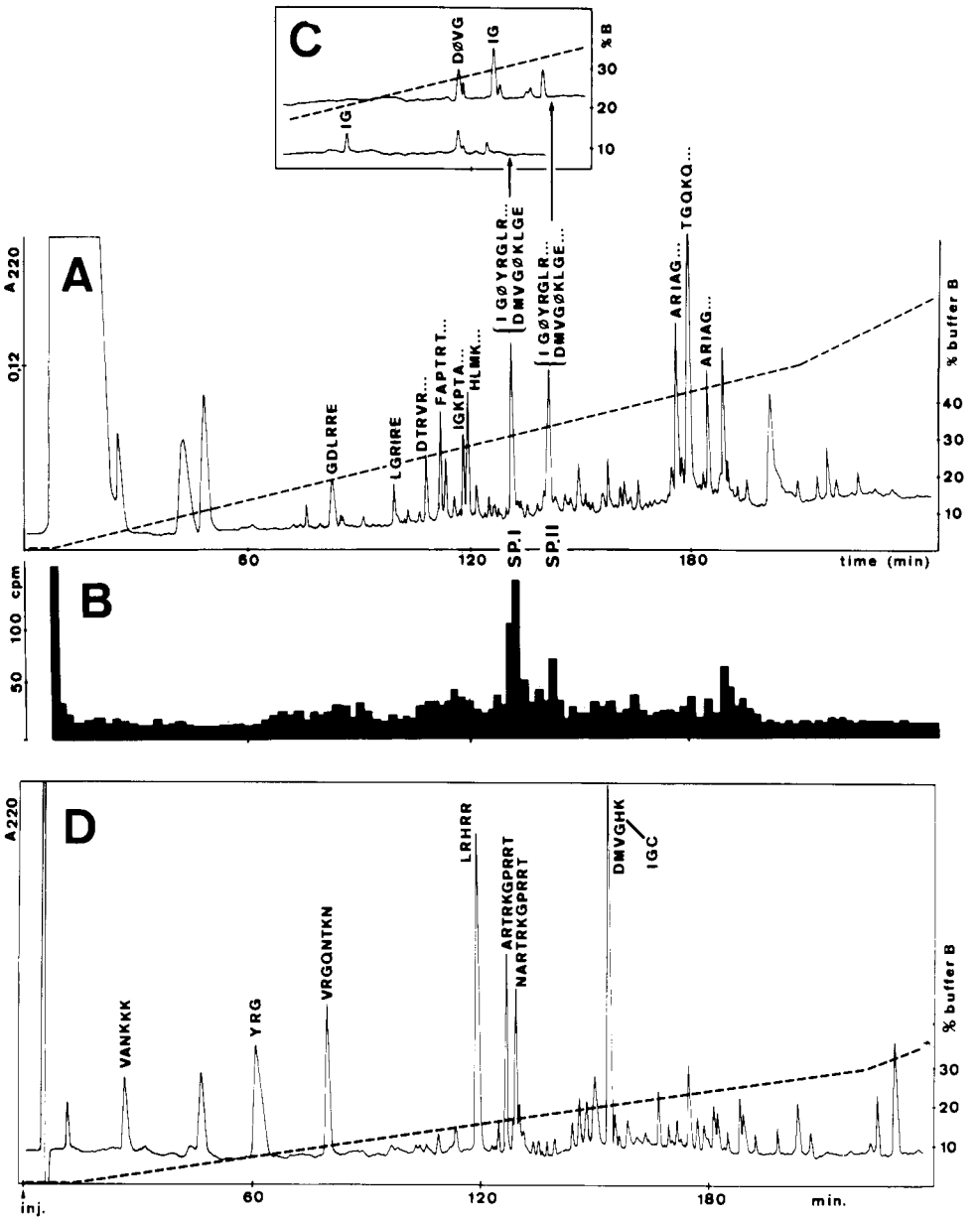

FIGURE 3: Isolation of cross-linked peptides derived from S13–S19. The chromatogram (A) shows the reversed-phase separation of the SP digest from 5 nmol of the protein pair S13–S19. The elution gradient is shown by the dashed line. The lower part of this figure (B) shows the radioactivity of the fractions of an identical separation of SP fragments of S13–S19 cross-link after reaction with tritiated diepoxybutane. For liquid scintillation counting, all fractions were dried in the counting vials, and then, 10 mL of the scintillation cocktail (premixed Beckman Ready-Solve MP) was added to each fraction. The upper inlay (C) shows rechromatographies of the two fractions with the highest radioactivity levels. These rechromatographies were done after periodate cleavage of SPI (lower trace) and SPII (upper trace). (D) presents the separation of thermolytic peptides of the large cross-linked peptide peak (SPI) of the upper chromatogram. The two cross-linked peptides are connected by a cross-bar between Cys and His. Detailed characterization of his double peptide, SP-Th, is given in the text and in Figure 5.

periodate cleavage and disappears after periodate oxidation forming two new bands in the low molecular weight region.] The cross-linked fragment (fraction 4 in Figure 2) was rechromatographed by reversed-phase HPLC (not shown) prior to and after periodate cleavage. By N-terminal sequencing of the fragments, the cross-linking site was found to be located within the two C-terminal fragments of proteins S13 and S19, starting with the sequences Glu-Ile-Gly (fragment of protein S13) and Val-Gly (fragment of protein S19). In the C-terminal parts of both proteins, many lysines are present, but none of these lysines was involved in this cross-link or even monovalently modified.

Staphylococcus aureus Protease Fragmentation and Recleavages. Application of ^{3}H-labeled diepoxybutane simplified the identification of SP peptides involved in the cross-linking bridge. As shown in Figure 3, only three significantly labeled peptides were obtained, the last of which represented the nondigested protein pair. The other two labeled fractions were cleaved with sodium periodate in order to distinguish cross-linked peptides from monovalently modified peptides. The periodate-treated fractions were rechromatographed under the same conditions as before. These chromatograms are shown in the insert of Figure 3. In both cases, the original peak disappeared and resulted in two new peaks of shorter retention times. In addition, amino acid compositions and N-terminal sequences were determined on the corresponding fractions prior to and after periodate cleavage. The cross-linked peptide fractions SPI and SPII both contained the same C-terminal S13 SP peptide spanning the region from Ile-81 to the C-terminus (see Figure 4) but had different-sized S19 fragments; SPI contained the S19 SP fragment from Asp-64 to Glu-72 and SPII the larger C-terminal S19 SP peptide from Asp-64

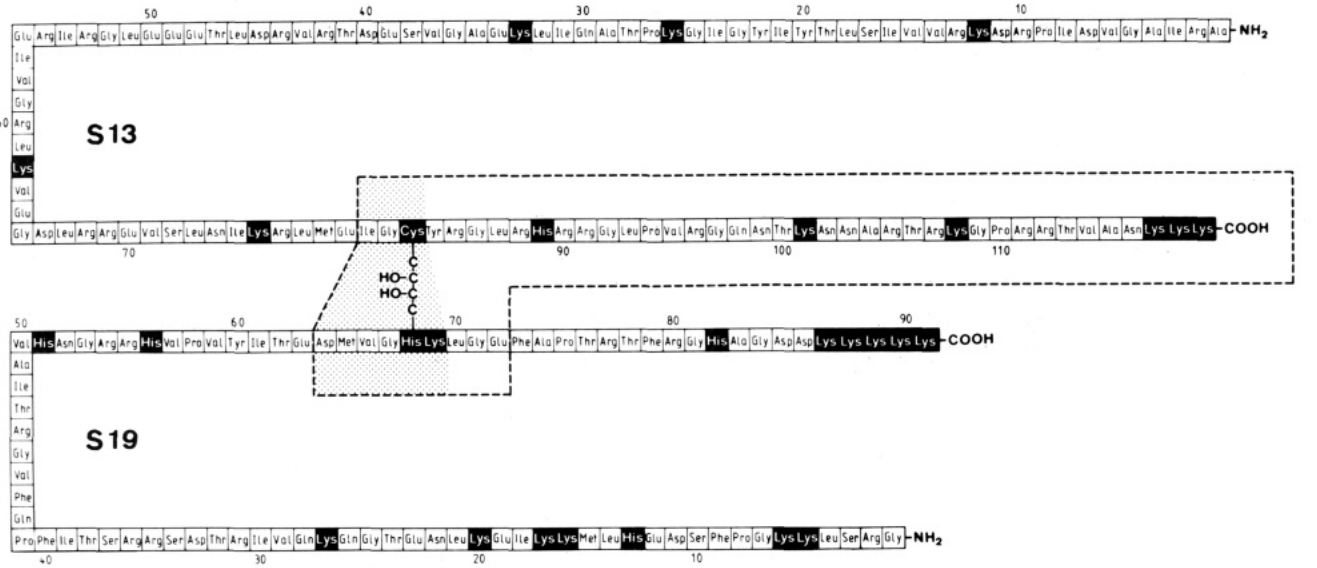

FIGURE 4: Cross-link site in the protein pair S13–S19. The complete sequences of proteins S13 and S19 are shown to illustrate the strategy of cross-linking site determination. Possible epoxide reactive residues are shown in black. The dashed line surrounds the two cross-linked SP peptides (SPI), while the spotted area marks the smallest isolated cross-linked peptide (SP-Th). Further cross-linked peptide fragments were isolated and confirm the same cross-link site, as described in the text.

to the C-terminus. Cross-linked peptide SPII derived from incomplete cleavage of glutamic acid at position 72. It can be concluded from this analysis that an amino acid between position 64 and position 72 of protein S19 is cross-linked to an amino acid within the C-terminal part of protein S13.

In order to determine the exact location within the C-terminal S13 fragment, the cross-linked fragment SPI was further digested with thermolysin. Separation of these peptides is shown in Figure 3. All fragments were sequenced manually, and only one double sequence was detected. Arrangement of these small peptides in the S13 and S19 protein sequences is shown in Figure 4. The manual liquid-phase sequence analysis of this peptide gave the following result:

	degradation step					
	1	2	3	4	5	6
S13 positions 81–83	Ile	Gly	X			
S19 positions 64–69	Asp	Met	Val	Gly	X	X

Neither cysteine-83 of protein S13 nor histidine-68 and lysine-69 of protein S19 could be identified. Amino acid analysis of this peptide, namely, SP-Th, gave the following composition: Asp, 0.95; Gly, 2.06; Met, 1.22; Val, 0.92; Ile, 1.03; Lys, 0.86. No further amino acid was detected in the hydrolysate.

Solid-phase sequencing after diisothiocyanate attachment yielded the same amino acids, except for the N-terminal residues, which are not detected due to DITC attachment (Figure 5A). However, solid-phase sequencing after attachment via the amino groups was only possible since coupling of the ϵ-amino group of lysine-69 of protein S19 kept the peptide covalently attached to the glass. Therefore, solid-phase sequencing proved that (1) both peptides were really cross-linked, because the S13 peptide alone, which contains no lysine, would only be coupled via the N-terminal α-amino group of the first amino acid by the DITC method but then be removed from the glass at the first cleavage reaction, and (2) histidine-68 and not lysine-69 is cross-linked to cysteine-83 in protein S13.

The cross-linking via histidine and cysteine is in good agreement with the amino acid analysis of this peptide pair (see above). Further evidence came from a peptide pair, cleaved by staphylococcal protease and trypsin, which eluted slightly later under the same HPLC conditions as used for the

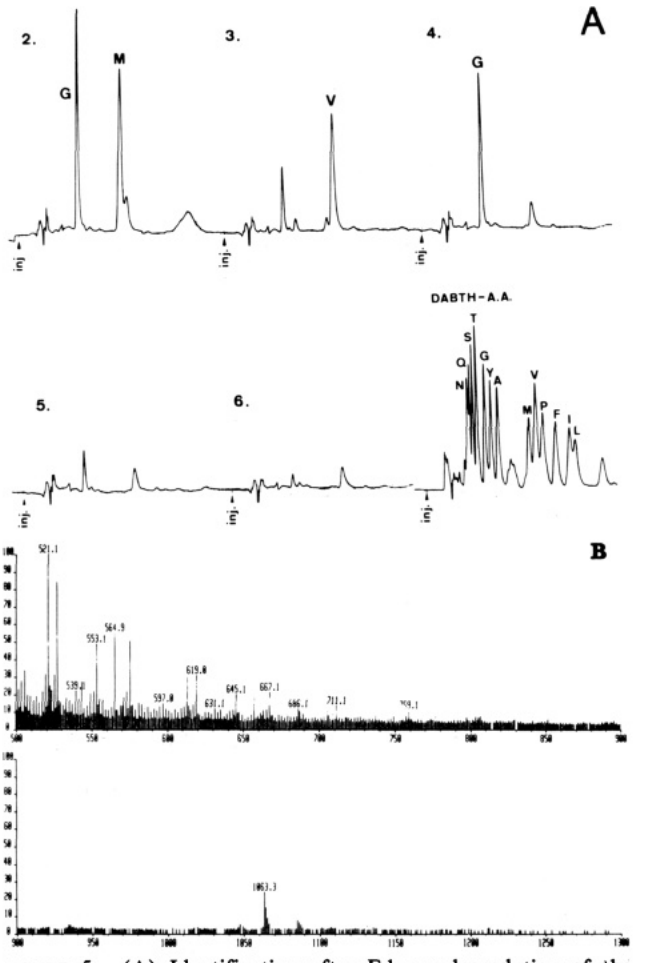

FIGURE 5: (A) Identification after Edman degradation of the cross-linked SP-Th peptides: Isocratic HPLC identification of DABTH amino acids released from cycle 2 to cycle 6 by solid-phase degradation of the peptide SP-Th, isolated as shown in Figure 3. With the DITC attachment to aminopropyl glass, both first residues are bound to the support and are therefore not identified (these residues were determined by manual liquid-phase DABITC microsequencing, as described in the text). (B) FAB mass spectrogram of the smallest cross-linked peptide (SP-Th) cleaved and isolated from the protein pair S13–S19, as shown in Figure 3. The mass ion $(M + H)^+$ of 1063 and a small $(M + Na)^+$ mass ion of 1085 are sample-derived signals.

Table I: Yields of PTH-Amino Acid Derivatives Released by Edman Degradation of Protein BST S13

cycle	amino acid	yield (pmol)	cycle	amino acid	yield (pmol)
1	Ala	580	20	Tyr	100
2	Arg	400	21	Ile	125
3	Ile	700	22	Tyr	115
4	Ala	460	23	Gly	110
5	Gly	390	24	Ile	110
6	Val	330	25	Gly	130
7	Asp	300	26	Lys	85
8	Ile	400	27	Pro	60
9	Pro	250	28	Thr	80
10	Arg	210	29	Ala	55
11	Asp	180	30	Gln	40
12	Lys	190	31	Ile	35
13	Arg	230	32	Leu	42
14	Val	160	33	Lys	40
15	Val	230	34	Glu	30
16	Ile	190	35	Ala	35
17	Ser	180	36	Gly	40
18	Leu	140	37	Val	18
19	Thr	160	38	Ser	25

peptide cleaved by thermolysin. This fragment contained the two cross-linked peptides Ile-Gly-X-Tyr-Arg and Asp-Met-Val-Gly-X-X. For additional confirmation of the results, the cross-linked SP-Th fragment was analyzed by fast atom bombardment mass spectrometry [for details see Eckart (1986)]. This analysis is shown in Figure 5B. The molecular ion signal $(M + H)^+$ of 1063.3 corresponds exactly to the mass of the two peptides Ile-Gly-Cys and Asp-Met-Val-Gly-His-Lys plus the mass of the butanediol bridge. The different analytical approaches applied to the analysis of the cross-linked protein–protein pair proves the single specific cross-linking site between cysteine-83 of protein S13 and histidine-68 of protein S19 in the *B. stearothermophilus* ribosome.

Furthermore, almost all other SP fragments of the cross-linked protein pair S13–S19 were sequenced as shown in Figure 3. It can be seen that most other parts of S13 and S19 can be recovered as single sequences. Many lysines of the cross-linked protein pair could be positively identified. This, together with the radioactive cross-linking reagent experiment shown in Figure 3, indicates that monovalent reaction of diepoxybutane with the lysines of proteins S13 and S19 under the conditions employed for the cross-linking reaction is not very frequent.

Sequence Determination of Protein S13 from B. stearothermophilus. Protein S13 from *B. stearothermophilus* was isolated by reversed-phase HPLC from the acetic acid protein extract of the 30S ribosomal subunit.

The N-terminal sequence of protein S13 up to position 38 was established by gas-phase microsequencing (see Table I). This N-terminal sequence confirms the first 15 amino acids of *B. stearothermophilus* protein S13 previously published (Yaguchi et al., 1974). The remaining sequence was determined by sequencing of various smaller sized peptides. The alignment of the complete set of staphylococcal protease peptides (for amino acid compositions, see Table II) was confirmed by sequencing of fragments obtained from digestion with lysyl-endopeptidase and clostripain (see Table III).

A single methionine residue was identified in position 79. The presence of one cysteine residue in protein S13 was determined by amino acid analysis after performic acid oxidation of the entire protein. This cysteine residue was identified at position 83 after carboxymethylation of protein S13 and clostripain digestion. In the chromatogram of peptides after this digestion of ^{14}C-carboxymethylated protein S13, only two fragments (CL8 and CL11; see Figures 6A and 7) showed radioactivity; this was due to the presence of (carboxymethyl)cysteine, as verified by amino acid analysis.

The C-terminal region of protein S13 is very basic, with 14 arginine and lysine residues, and contains no acidic amino acid. The determination of the number of consecutive lysines in the C-terminus caused problems because of overlap in degradation and wash-out of the small remaining lysine peptide. Unambiguous determination of three lysine residues was possible by solid-phase sequencing of the C-terminal thermolytic peptide after carboxyl attachment to aminopropyl glass (see Table IV). Finally, the C-terminal thermolytic peptide was analyzed by fast atom bombardment mass spectrometry, which confirmed the presence of three lysines. Surprisingly, a staphylococcus protease cleavage was obtained reproducibly between glycine-23 and isoleucine-24. This may be explained by thermolysin-like impurities in the commercial enzyme. Complete sequence determination by use of several independent sets of peptides was possible as shown in Figure 7. The amino acid composition derived from this sequence agrees well with results obtained from amino acid analysis of the entire protein as shown in Table V. On the basis of sequence, the molecular mass of protein S13 of *B. stearothermophilus* is 13 556.

The following characteristic structural features of ribosomal protein S13 can be deduced from the primary structure: Two very basic areas occur at the N- and C-terminal parts of the basic protein S13. The basic C-terminal region contains clusters of arginines and lysines. The middle region of the protein has several acidic residues together with basic residues

Table II: Amino Acid Composition of *Staphylococcus* Protease Fragments of Protein S13 from *B. stearothermophilus* Determined by the OPA Precolumn Derivatization Procedure

	SP1	SP2	SP3	SP4	SP6	SP7	SP9	SP11	SP12
Asx		2.21 (2)	1.05 (1)				4.5 (4)	1.09 (1)	1.62 (2)
Glx	0.95 (1)	3.00 (3)	0.99 (1)	1.01 (1)	1.01 (1)	1.92 (2)	1.11 (1)	1.0 (1)	
Ser	0.68 (1)							0.67 (1)	0.76 (1)
His							1.02 (1)		
Gly	1.1 (1)		1.16 (1)	1.13 (1)	1.10 (1)	1.09 (1)	5.7 (5)		2.95 (2)
Thr		1.6 (2)				0.67 (1)	2.64 (3)		0.79 (1)
Arg		1.97 (2)	1.94 (2)	2.00 (2)	0.92 (1)		9.3 (9)	1.02 (1)	3.37 (3)
Ala	1.13 (1)					0.86 (1)	2.39 (2)		2.3 (2)
Tyr							1.13 (1)		1.9 (2)
Met								0.94 (1)	
Val	1.14 (1)	1.25 (1)			1.98 (2)	0.78 (1)	2.23 (2)	1.26 (1)	3.11 (3)
Phe									
Ile				0.89 (1)	0.79 (1)	1.65 (2)	0.92 (1)	0.88 (1)	3.41 (4)
Leu		0.98 (1)	0.87 (1)	0.97 (1)	0.96 (1)	1.08 (1)	2.02 (2)	1.89 (2)	1.07 (1)
Lys					1.23 (1)	2.95 (2)	6.43 (5)	1.24 (1)	1.74 (1)
Pro[a]									

[a] Not determined.

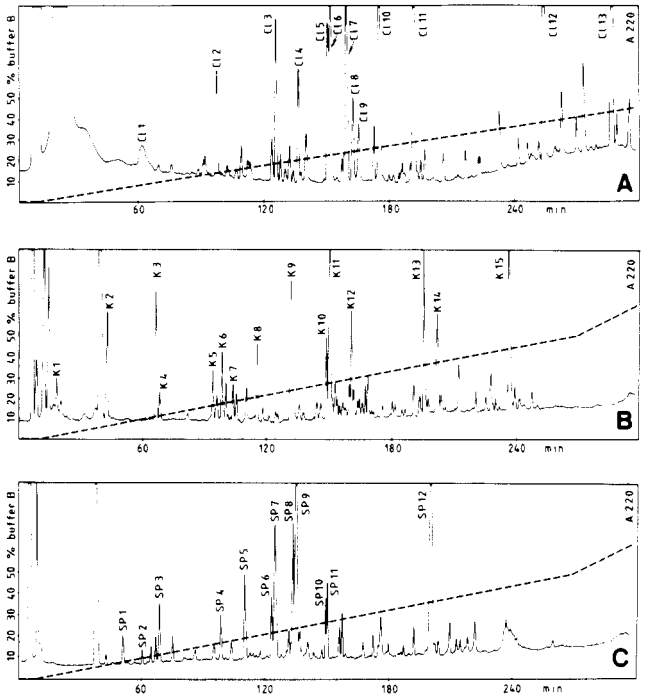

FIGURE 6: Reversed-phase HPLC separations of S13 peptides, used for sequence determination of protein S13. Peptides were separated on a TSK ODS 120T column. Buffer A was 0.1% aqueous TFA; buffer B was 80% acetonitrile and 0.1% TFA. Gradients are indicated by the dotted lines. Flow rate was 0.7 mL/min, and column temperature was 35 °C. UV absorption was measured at 220 nm with 0.16 absorption unit full scale. (A) shows fragments after clostripain digestion of protein BST S13, (B) shows fragments after cleavage of protein S13 with lysyl-endopeptidase, and (C) shows peptides obtained after *S. aureus* protease treatment. A total of 6 nmol of protein S13 was digested.

and contains the single cysteine residue (position 83) of the protein. Near to this the single methionine (position 79) and histidine (position 89) residues are found.

On the basis of the primary structure, predictions of the secondary structure were made by employing four different algorithms. The prediction results are shown in Figure 8. Notable are three calculated α-helical regions (positions 30–35, 46–52, and 114–119), one region of β-sheet structure (positions 13–20), and one β-turn region (positions 84–111). Within this predicted β-turn area, the single cysteine is located, which is cross-linked to protein S19, as identified in this study.

Table III: Yields of PTH-Amino Acids Released by Gas-Phase Degradation of Peptide K15 (Positions 34–62)

cycle	amino acid	yield (nmol)	cycle	amino acid	yield (nmol)
1	Glu	*a*	16	Glu	1.6
2	Ala	*a*	17	Glu	1.4
3	Gly	3.9	18	Leu	0.9
4	Val	3.7	19	Gly	1.2
5	Ser	3.6	20	Arg	1.3
6	Glu	3.6	21	Ile	0.75
7	Asp	3.7	22	Arg	1.3
8	Thr	4.0	23	Glu	0.6
9	Arg	3.5	24	Ole	0.55
10	Val	2.4	25	Val	0.48
11	Arg	3.0	26	Gly	0.6
12	Asp	2.5	27	Arg	0.63
13	Leu	1.5	28	Leu	0.25
14	Thr	2.6	29	Lys	0.09
15	Glu	1.5			

[a] No quantitation, because the first two steps were degraded by the manual DABITC–PITC method, as described in the text.

Table IV: Yields of PTH-Amino Acids Released by Solid-Phase Degradation of the C-Terminal Fragment Th6 of Protein BST S13 (Positions 114–119)

cycle	amino acid	yield (pmol)
1	Val	210
2	Ala	200
3	Asn	105
4	Lys	143
5	Lys	127
6	Lys	125
7	(Lys overlap)	(29)

The sequence of protein S13 was compared with those of all other sequenced ribosomal proteins of different sources for identical and homologous sequences with the computer programs ALIGN (Wittmann-Liebold et al., 1984) and PRETTY (UWGCG program). Only one homologous protein was found after comparison with all known ribosomal protein sequences, namely, protein S13 of *E. coli* (Lindemann et al., 1976). The sequence comparison is presented in Figure 9 and results in 58% identical residues at almost identical positions (including one gap for one amino acid).

DISCUSSION

The protein pair S13–S19 produced by cross-linking with diepoxybutane showed high stability during isolation and

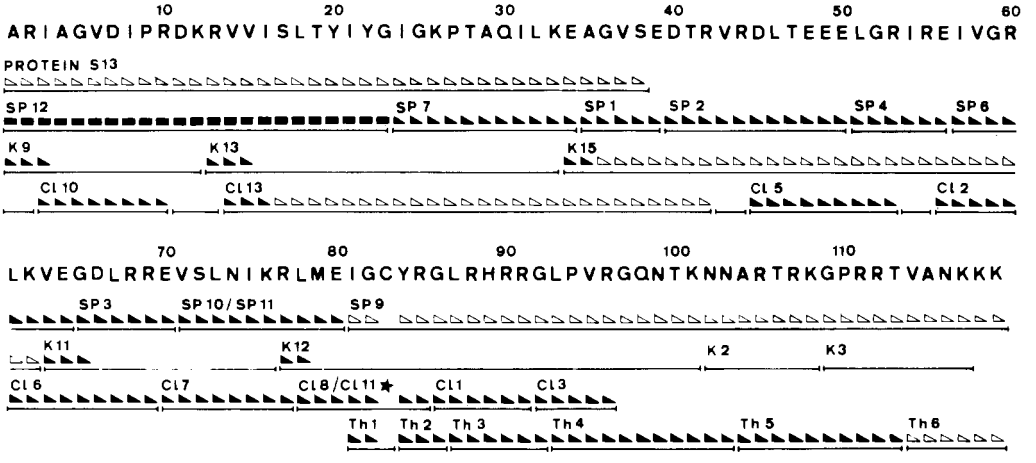

FIGURE 7: Strategy for sequence determination of protein S13 from *B. stearothermophilus*. Symbols: (open triangle) residue determined by gas-phase sequencing; (black triangle) residue determined by manual DABITC–PITC microsequencing; (black rectangle) residue determined by automatic DABITC–PITC solid-phase sequencing. SP, K, Cl, and Th refers to S13 fragments derived from digestion with *S. aureus* protease, lysyl-endopeptidase, clostripain, and thermolysin. The asterisk marks the clostripain fragments, which were radioactively labeled after ^{14}C carboxymethylation.

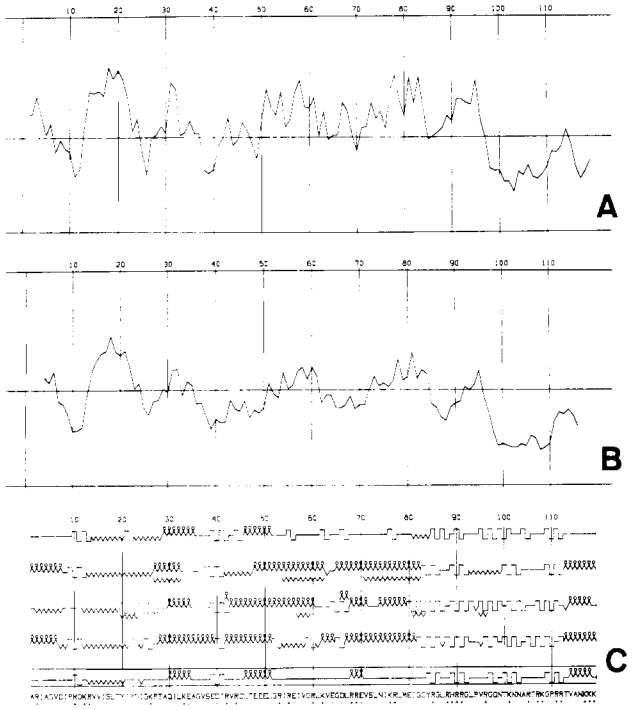

FIGURE 8: (A) Hydropathy plot of protein BST S13 according to the algorithms of Nishkawa and Ooi (1980). (B) Hydropathy plot according to Kyte and Doolittle (1982). (C) Predicted secondary structure for protein S13 from *B. stearothermophilus* according to four different algorithms as described in Rawlings et al. (1983). Loops represent α-helix, zig-zags represents β-sheets, and steps indicate β-turns; straight lines represent random coil.

during all procedures required for identification of the cross-linking site. The cross-linked proteins and peptides were easily cleavage by mild specific periodate oxidation. This facilitated the identification of cross-linked proteins and peptides at all stages of the isolation of cross-linked proteins and peptides. Diepoxybutane generated only a few protein pairs, some of which were found in relatively high yield. Therefore, the reagent proved to be suitable for this type of analysis, which requires isolation of cross-linked proteins on a preparative scale. The short reagent length and its water solubility are further important advantages of diepoxybutane.

In this study, the type of amino acid which was cross-linked was determined. We expected that mainly lysines would become cross-linked. However, none of the many lysines present

Table V: Amino Acid Composition of Protein S13 from *B. stearothermophilus*

	from sequence	determined by the OPA method
Cys	1	1[a]
Asp/Asn	5/5	10.3
Glu/Gln	9/2	11.7
Ser	3	2.3
His	1	1.3
Gly	12	10.3
Thr	7	7.0
Arg	20	22.3
Ala	6	6.2
Tyr	3	3.4
Trp	0	0
Met	1	0.7
Val	10	8.6
Phe	0	0
Ile	10	9.6
Leu	10	10.1
Lys	10	10.2
Pro	4	nd[b]
Σ	119	115 (+4 Pro)

[a] Determined as cysteic acid after performic acid oxidation. [b] Not determined.

in both proteins had reacted with diepoxybutane to form cross-links. The sequence analysis of these cross-linked proteins and peptides gave no evidence for substantial monovalent modification of the lysines. This results differs from the earlier study of Allen et al. (1979), where some residues in proteins S5 and S8 were significantly modified by the reagent dimethyl suberimidate. Obviously, the reactivity of diepoxybutane toward the ϵ-amino groups is rather low since the reaction with diepoxybutane is performed at pH 7.9 where most of the ϵ-amino groups of the lysines are still protonated. (The pK value of lysine side chain amino groups is 10.5.) Furthermore, the lysines may in part be located in the interior of the ribosome and therefore are not accessible to the reagent.

In this study, tritiated diepoxybutane was applied for cross-linking and made location of the cross-linked peptides easier during the purification steps. The value of using radiolabeled cross-linking agents seems limited however, since any monovalent modification of the amino acids results in a raise of radioactivity in the peptide chromatograms. Nevertheless, use of radioactively labeled reagent supported the results from the isolation as demonstrated. For an unambiguous determination of the cross-linked residues, comple-

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                    10                  20                  30
ECO. S13:  A R I A G I N I P D H K H A V I A L T S I Y G V G K T R S K
BST. S13:  A R I A G V D I P R D K R V V I S L T Y I Y G I G K P - T A

                    40                  50                  60
ECO. S13:  A I L A A A G I A E D V K I S E L S E G Q I D T L R D E V A
BST. S13:  Q I L K E A G V S E D T R V R D L T E E E L G R I R E I V G

                    70                  80                  90
ECO. S13:  K F V V E G D L R R E I S M S I K R L M D L G C Y R G L R H
BST. S13:  R L K V E G D L R R E V S L N I K R L M E I G C Y R G L R H

                   100                 110
ECO. S13:  R R G L P V R G Q R T K T N A R T R K G P R K P I K K
BST. S13:  R R G L P V R G Q N T K N N A R T R K G P R R T V A N K K K
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FIGURE 9: Amino acid sequence comparison of ribosomal proteins S13 of *B. stearothermophilus* and *E. coli*.

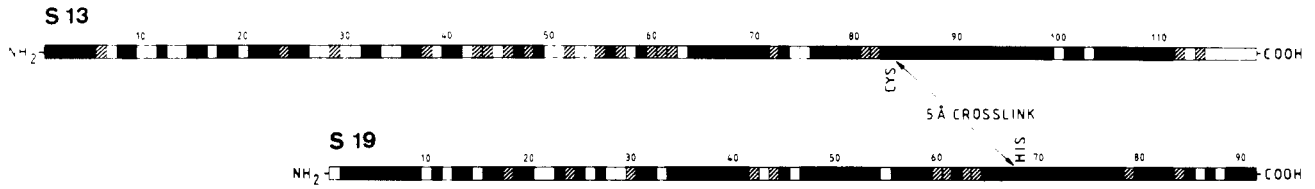

FIGURE 10: Schematic representation of protein BST S13 and BST S19 sequence homologies with the respective proteins derived from *E. coli* ribosomes. Black boxes indicate identical residues in both proteins. Striped areas refer to conservative amino acid replacements (K/R, I/L, L/V, I/V, S/T, A/G, Q/N, E/D, F/Y). The high sequence conservation in the C-terminal half of the proteins is evident.

mentary techniques such as HPLC prior to and after periodate cleavage, polyacrylamide gel electrophoresis, diagonal gel electrophoresis, sequence analysis, and FAB mass spectrometry were necessary.

Mass spectrometry of the smallest cross-linked peptide proved that the bridge in the cross-link was formed solely by one molecule of the reagent and not by an adduct of more than one reagent molecule (which is a more severe problem with other reagents, such as suberimidates, rather than with diepoxybutane).

Many protein neighborhoods within the *E. coli* ribosome have been determined on the protein level, but only in two instances have the cross-linking sites been identified at the amino acid level. This investigation demonstrates that the amino acid cysteine of protein S13 is cross-linked by diepoxybutane to the histidine residue of position 68 in protein S19. Due to the small reagent applied, the distance between these two amino acids within the ribosome is 5 Å or less.

Comparison of this result with neutron scattering studies of the *E. coli* ribosome shows some discrepancies. The distance between the centers of mass of proteins S13 and S19 was determined to be 85 Å, and a distance of 40 Å was given as closest approach (Moore et al., 1986). According to our cross-linking experiments, this distance is extremely unlikely and should be remeasured. Proteins S13 and S19 of the *E. coli* ribosome were identified as neighbors by earlier cross-linking studies using the reagents dimethyl suberimidate (Expert-Bezançon et al., 1976), tartryl diazide (Lutter et al., 1975), iminothiolane (Lambert et al., 1983), and diepoxybutane (Pohl et al., 1986). Also, immune electron microscopy studies revealed that both proteins are located close together on the "head" of the small ribosomal subunit (Stöffler et al., 1986).

It was shown earlier that individually neither S13 nor S19 in the *E. coli* system binds to 16S RNA but together a rather strong binding was observed (Dijk et al., 1977). Furthermore, both proteins could be cross-linked with the initiation factor IF-3 (Pon et al., 1982), which indicates that proteins S13 and S19 are involved in the initiation process.

A schematic comparison of the complete amino acid sequences of proteins S13 and S19 from *B. stearothermophilus* and *E. coli* is presented in Figure 10. The cross-link is clearly located in the most conserved regions of both proteins. This fact suggests that this region in particular has an important functional role. A single cysteine residue and histidine, methionine, and tyrosine residues were identified in the contact areas. Cysteine-83 is likely to have an exposed position as indicated by the predicted β-turn structure in this part of the sequence (Figure 9). The importance of these residues for ribosomal function is indicated by some other studies. For instance, the tRNA binding capability of elongation factor EF-Tu was abolished by modification of a single cysteine residue and drastically reduced by histidine modification (Jonák et al., 1980). The binding capacity of initiation factor IF-3 to the 30S subunit can be greatly reduced through iodination of tyrosines (Bruhns et al., 1980) or modification of certain lysines (Ohsawa et al., 1981).

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Purification and Properties of an 18-Kilodalton, 1,25-Dihydroxyvitamin D_3 Modulated Protein from Embryonic Chick Intestine†

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ABSTRACT: An 18 000-dalton protein (p*I* = 5.1) shown previously to be modulated by 1,25-dihydroxyvitamin D_3 was purified to allow its further characterization. This protein from embryonic chick intestine was shown to comigrate during two-dimensional electrophoresis with an abundant protein from the intestine of 4-week-old chickens. The protein was purified from 4-week chick intestine and analyzed for amino acid composition, and 28 amino acids of its N-terminal sequence were determined. The N-terminal amino acid sequence had significant homology to cellular retinol binding protein II, an intestinal protein that has been recently sequenced. The purified 18-kilodalton protein was shown to bind retinol by fluorescence spectrophotometry. This 18-kilodalton protein is dramatically changed by 1,25-dihydroxyvitamin D_3 in the chick embryonic organ culture system. Therefore, further study of it may lead to a better understanding of vitamin A and D interaction and how 1,25-dihydroxyvitamin D_3 acts through proteins to stimulate intestinal calcium and phosphate transport.

The primary function of vitamin D is to regulate blood calcium and phosphate concentrations. The hormonally active form of the vitamin, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] plays its most important role in this process by stimulation of intestinal calcium and phosphate transport (DeLuca & Schnoes, 1984; DeLuca, 1986). We are interested in determining the molecular mechanism whereby 1,25-$(OH)_2D_3$ stimulates calcium and phosphate transport. To date, this mechanism is still unknown.

The chick embryonic duodenal organ culture (EDOC) is an ideal system in which to study the modulation of intestinal proteins by 1,25-$(OH)_2D_3$. In this system, it is possible to label

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; EDOC, embryonic duodenal organ culture; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; kDa, kilodalton; CaBP, vitamin D dependent calcium binding protein; p*I*, isoelectric point; M_r, molecular weight; CRBP II, cellular retinol binding protein II; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; DEAE, diethylaminoethyl; 2-ME, 2-mercaptoethanol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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