Identification of Cross-Linked Amino Acids in the Protein Pair HmaL23–HmaL29 from the 50S Ribosomal Subunit of the Archaebacterium *Haloarcula marismortui*

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ABSTRACT: 50S ribosomal subunits from the extreme halophilic archaebacterium *Haloarcula marismortui* were treated with the homobifunctional protein–protein cross-linking reagents diepoxybutane (4 Å) and dithiobis(succinimidyl propionate) (12 Å). The dominant product with both cross-linking reagents was identified on the protein level as HmaL23–HmaL29, which is homologous to the protein pair L23–L29 from *Escherichia coli* [Walleczek, J., Martin, T., Redl, B., Stöffler-Meilicke, M., & Stöffler, G. (1989) *Biochemistry 28*, 4099–4105] and from *Bacillus stearothermophilus* [Brockmöller, J., & Kamp, R. M. (1986) *Biol. Chem. Hoppe-Seyler 367*, 925–935]. To reveal the exact cross-linking site in HmaL23–HmaL29, the cross-linked complex was purified on a preparative scale by conventional and high-performance liquid chromatography. After endoproteolytic fragmentation of the protein pair, the amino acids engaged in cross-link formation were unambiguously identified by N-terminal sequence analysis and mass spectrometry of the cross-linked peptides. The cross-link is formed between lysine-57 in the C-terminal region of HmaL29 and the α-amino group of the N-terminal serine in protein HmaL23, irrespective of the cross-linking reagent. This result demonstrates that the N-terminal region of protein HmaL23 and the C-terminal domain of HmaL29 are highly flexible so that the distance between the two polypeptide chains can vary by at least 8 Å. Comparison of our cross-linking results with those obtained with *B. stearothermophilus* revealed that the fine structure within this ribosomal domain is at least partially conserved.

Protein biosynthesis on the ribosome is one of the most fundamental processes in each living cell. In order to understand the mechanism of translation at the molecular level, many experimental approaches have been made to elucidate the topography of ribosomal components and to construct a consistent model of the ribosomal particles. Among these, protein–protein cross-linking has found wide application to establish protein neighborhoods in the eubacterial as well as in the eukaryotic ribosome (Wallezcek et al., 1989; Uchiumi et al., 1985; Xiang & Lee, 1989). Fine structure information was obtained by extending this structure analysis to the determination of the amino acids involved in cross-link formation in the protein pair S5–S8 (Allen et al., 1979) and within a pair of the L7/L12 proteins (Maassen et al., 1981). As was shown for the protein pair S13–S19, identical cross-linking sites were generated in *Escherichia coli* and in *Bacillus stearothermophilus* (Pohl & Wittmann-Liebold, 1988; Brockmöller & Kamp, 1988), thereby identifying highly conserved structural domains in ribosomes from different eubacterial organisms.

Woese and Fox (1977) showed that archaebacteria can be regarded as a third kingdom in addition to eubacteria and eukaryotic organisms. Comparison of primary structures of ribosomal proteins from the archaebacterium *Haloarcula marismortui* with those of eubacteria and eukaryotes revealed that many proteins have equivalents in all organisms with homologous primary structures whereas others do not show significant sequence similarities (Wittmann-Liebold et al., 1990; Arndt et al., 1991). In the conserved proteins, the archaebacterial sequences form the link between eubacterial and eukaryotic ribosomes (Wittmann-Liebold, 1988). Therefore, we are investigating whether or not structural similarities between eubacterial and archaebacterial ribosomes are also expressed in the three-dimensional arrangement of ribosomal proteins. To this end, we have initiated an extensive protein–protein cross-linking study in 50S ribosomal subunits from *Haloarcula marismortui* using the homobifunctional cross-linking reagents diepoxybutane (DEB)[1] and dithiobis(succinimidyl propionate) (DSP). These reagents have been proven to be very useful concerning their reactivity with the ribosome under native conditions and their stability during the purification of the cross-linked protein pairs (Kamp, 1988). Both reagents react with ϵ-amino groups of lysines or the α-amino group of the N-terminal amino acid. As was shown by Pohl and Wittmann-Liebold (1988) and by Brockmöller and Kamp (1988), DEB also reacts with cysteine and histidine under native pH conditions.

Several cross-linked complexes in *H. marismortui* have been obtained using these two reagents (Bergmann, 1990; Bergmann, unpublished experiments). The monomeric proteins have been unequivocally identified by 2D-PAGE and protein sequencing. In this paper, we describe the purification and identification of the protein pair HmaL23–HmaL29, the dominant cross-link formed both by DEB and by DSP. The homologous protein pair has previously been described in cross-linking studies of *E. coli* (Walleczek et al., 1989). Recently, the same cross-link has been obtained using DEB in 50S ribosomal subunits from *B. stearothermophilus* (Brockmöller & Kamp, 1986), and the cross-linked amino acids have been determined (S. Herwig, V. Kruft, K. Eckart, and B. Wittmann-

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[1] Abbreviations: DEB, diepoxybutane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DSP, dithiobis(succinimidyl propionate); HmaL23–HmaL29DEB, protein pair HmaL23–HmaL29 cross-linked with DEB; PAGE, polyacrylamide gel electrophoresis; *o*-PDM, *o*-phenylenedimaleimide; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TP50, total protein of the 50S ribosomal subunit.

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Liebold, *J. Biol. Chem.*, in press). In order to compare a possible conservation of this cross-link on the amino acid level, we have extended our study to the assignment of the cross-linked residues in HmaL23–HmaL29. Furthermore, structural aspects concerning the localization of the two proteins within the 50S subunit will be discussed.

MATERIALS AND METHODS

Chemicals. DL-1,2:3,4-Diepoxybutane was purchased from Serva (Heidelberg, Germany). Dithiobis(succinimidyl propionate) was from Pierce (Rockford, IL), sodium metaperiodate was from Fluka (Switzerland), and methylamine was obtained from Sigma (Deisenhofen, Germany).

Lysylendoproteinase (LysC) was purchased from Boehringer Mannheim (Germany), chymotrypsin was from Merck (Darmstadt, Germany), and thermolysin was from Serva.

Acrylamide (2× crystallized) was purchased from Serva and *N,N'*-methylenebis(acrylamide) (Ultra Pure) was from BRL (Gaithersburg, MD).

2-Propanol and acetonitrile were LiChrosolv grade, and *N,N*-dimethylformamide was Uvasol quality obtained from Merck. The reagents used for automated protein sequencing on the pulsed-liquid-phase sequenator (Model 477A; Applied Biosystems, Foster City, CA) were purchased from the manufacturer. The solvents were Fluka sequencer grade, purified over Al_2O_3 under nitrogen and redistilled under nitrogen.

HPLC columns for protein purification (4 mm × 250 mm) were laboratory-packed with Vydac C4 material (5-μm particle size and 300-Å pore diameter) which was obtained from The Separation Group (Hesperia, CA). HPLC support for peptide separation was LiChrospher RP18 (5 μm, 100 Å), purchased as a prepacked cartridge (4 mm × 250 mm) from Merck.

Preparation of Ribosomes. Growth of *H. marismortui* cells and preparation of ribosomes and ribosomal subunits were done as described by Shevack et al. (1985). Ribosomal proteins were extracted by acetic acid treatment according to Hardy et al. (1969) under the modified conditions described earlier (Bergmann & Arndt, 1990).

Cross-Linking with DEB. 50S ribosomal subunits were dialyzed overnight into a buffer containing 2.7 M KCl, 50 mM $MgCl_2$, and 200 mM triethanolamine hydrochloride, pH 8, and diluted to a concentration of 50 A_{260}/mL. DEB was added to a final concentration of 2%, and the reaction mixture was stirred for 2 h at ambient temperature. Cross-linking was stopped by the addition of one-third of the volume of a 2 M methylamine solution, within a 30-min period. Ribosomal subunits were separated from excess reagent by ultracentrifugation in a Beckman 60Ti rotor at 9 °C and 42 000 rpm for 22 h and subsequently subjected to protein extraction.

Periodate Cleavage. Cross-linked proteins were dissolved in 20 μL of 0.1% aqueous TFA and cleaved by the addition of 10 mM sodium metaperiodate within 15–30 min at ambient temperature. Oxidation was stopped by direct injection onto HPLC columns or by lyophilization prior to electrophoresis.

Cross-Linking with DSP. 50S ribosomal subunits were extensively dialyzed into a buffer containing 2.7 M KCl, 50 mM $MgCl_2$, and 50 mM triethanolamine hydrochloride, pH 8, and brought to a concentration of 50 A_{260}/mL. DSP was freshly dissolved in DMSO to a concentration of 20 mg/mL and added to the prechilled ribosome solution to give a final concentration of 1 mM. Cross-linking reactions were allowed to take place for 2 h at 4 °C under stirring. Then the temperature was elevated to 37 °C and maintained for a further 60 min. The reaction was stopped by the addition of 1% 5 M NH_4Cl and a further incubation for 40 min. Ribosomal subunits were recovered as described above.

Cleavage of DSP–Disulfide Bridges. Cross-linked proteins were dissolved in 50 mM Tris-HCl, pH 8, and the reagent was cleaved by the addition of 30 mM β-mercaptoethanol. The reaction occurred for 2 h at 37 °C, and samples were directly subjected to HPLC. Alternatively, cleavage was performed in 25 μL of 1D or SDS sample buffer prior to electrophoresis (see below).

Ion-Exchange Chromatography. Conventional ion-exchange chromatography on DEAE-cellulose DE52 (Whatman), column size 2.5 cm × 25 cm, was carried out essentially as described by Hatakeyama and Kimura (1988) except that the extracted and lyophilized proteins were dissolved in 80 mM Tris–citrate, pH 8.3, containing 4 M urea and were stepwise dialyzed into equilibration buffer, namely, 80 mM Tris–citrate, pH 8.3, containing 30% DMF. Proteins were eluted at a flow rate of 2.5 mL/min with a gradient of 0–300 mM KCl in equilibration buffer. Fractions of 15 mL were collected, and every third fraction was analyzed by SDS–PAGE. In order to remove the salt, the proteins were precipitated for 2 h at –20 °C by 12% trichloroacetic acid in the presence of 200 μg/mL deoxydodecylcholate. The protein pellets were washed twice with acetone, dried, and dissolved in SDS sample buffer. Larger volumes of pooled fractions were desalted by RP-HPLC, as described previously (Pohl & Kamp, 1987).

HPLC of Proteins and Peptides. Proteins were dissolved in 5% acetic acid and separated on a Vydac C4 column using a gradient made of 0.1% aqueous TFA (buffer A) and 0.1% TFA in 2-propanol (buffer B) at a flow rate of 0.5 mL/min at 40 °C. Separation of peptides was performed on LiChrospher RP-18 with a gradient made of buffer A and 0.1% TFA in acetonitrile as buffer B. The elution of proteins and peptides was monitored at 220 nm.

Polyacrylamide Gel Electrophoresis. One-dimensional SDS–PAGE was carried out as described by Fling and Gregerson (1986). Two-dimensional PAGE was performed according to Hatakeyama and Kimura (1988).

Enzymatic Cleavages. Fragmentation of the purified cross-link with lysylendoproteinase (LysC) was carried out in 100 mM NH_3HCO_3, pH 8.9, in the presence of 4 M urea at 37 °C for 16 h and at an enzyme:substrate ratio of 0.1 unit of enzyme/100 μg of protein. Cleavage with chymotrypsin occurred in 100 mM *N*-methylmorpholine acetate, pH 8.1, at 37 °C in 4 h and an enzyme:substrate ratio of 1:50. Enzymatic digestion with thermolysin was done in 100 mM *N*-methylmorpholine acetate, pH 8.1, at 50 °C for 2 h and an enzyme:substrate ratio of 1:50. All digestions were stopped by direct injection into HPLC.

Microsequencing. Microsequencing of the proteins and of the cross-linked protein pairs and peptides was done with polybrene on glass filters in the ABS sequencer.

Plasma Desorption Mass Spectrometry. Molecular masses of the peptides were determined with the BioIon mass spectrometer from Applied Biosystems; 10–50 pmol of the sample dissolved in 20 μL of 0.1% TFA in 20% acetonitrile was analyzed under standard conditions.

RESULTS

Cross-Linking of 50S Subunits. 50S ribosomal subunits of *H. marismortui* were reacted under native conditions with either DSP or DEB. The proteins were extracted by acetic acid/Mg^{2+} and analyzed by 2D-PAGE (Figure 1). Cross-linked protein pairs appear in the electrophoretogram as

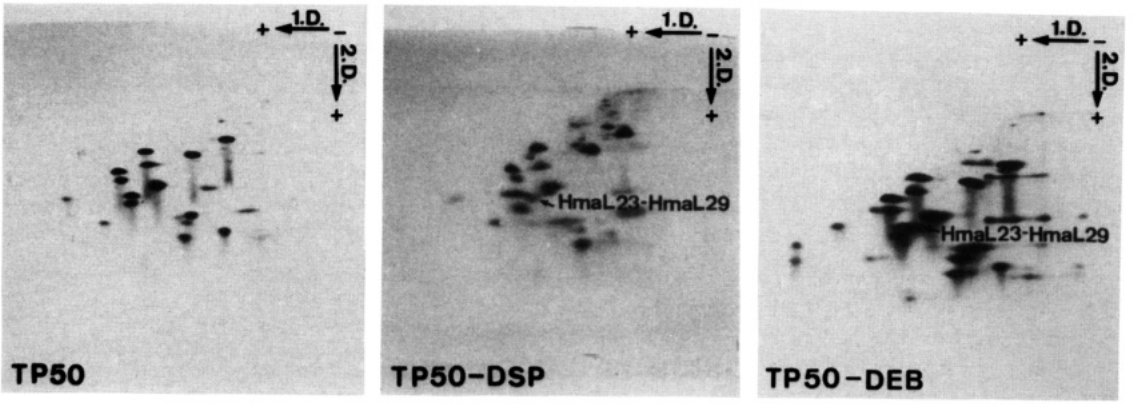

FIGURE 1: 2D-PAGE analysis of cross-linked 50S subunits from *H. marismortui*. Proteins of untreated (TP50) and DSP cross-linked (TP50–DSP) and DEB cross-linked (TP50–DEB) 50S subunits were extracted and subjected to electrophoresis. The position of the cross-link HmaL23–HmaL29 is indicated in the electrophoretogram.

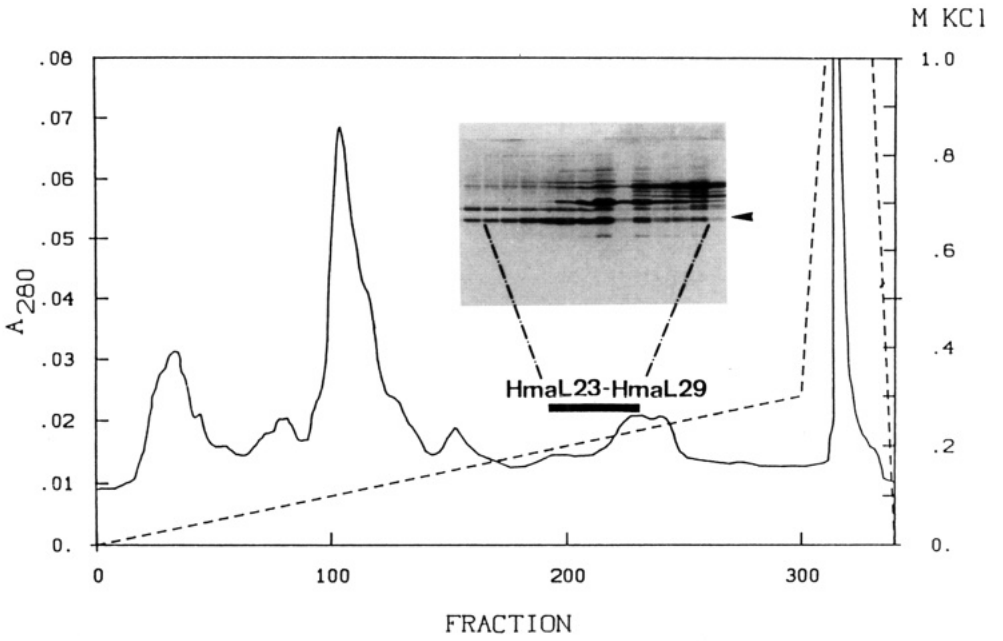

FIGURE 2: Purification of HmaL23–HmaL29DSP by ion-exchange chromatography on DEAE-cellulose DE52. Approximately 400 mg of protein mixture was applied to the column and eluted by a linear KCl gradient (dashed line). The flow rate was 2.5 mL, and fractions of 15 mL were collected. Elution of HmaL23–HmaL29DSP was monitored at 280 nm. The insert shows the cross-link-containing fractions which were analyzed by SDS–PAGE.

additional spots compared to the regular 50S protein pattern and disappear upon cleavage of the cross-link bridge with β-mercaptoethanol or periodate, respectively (not shown). HmaL23–HmaL29 was formed as the major cross-linked complex in both cross-linking reactions. The yield of the protein pair was estimated between 10 and 20% through comparison of the intensity of the protein spot in the acrylamide gel. The amounts of HmaL23–HmaL29 were comparable for DSP and DEB and were found to be reproducible over several cross-linking reactions.

The purification steps of the protein pair were identical irrespective of the reagent used and will be illustrated in the case of the DSP cross-link. The determination of the exact cross-linking site by N-terminal sequence analysis and mass spectrometry is given for each protein pair.

Preparative Isolation of HmaL23–HmaL29DSP. Purification of HmaL23–HmaL29DSP after cross-linking with DSP in a preparative scale was accomplished by two-step chromatography starting with conventional open-column ion-exchange chromatography on DEAE-cellulose DE52. In a typical preparation, 400–600 mg of cross-linked TP50 was applied to the column and chromatographed using a KCl gradient ranging from 0 to 300 mM KCl. The elution of the protein was followed spectrophotometrically at 280 nm and by SDS–PAGE of the resulting fractions (Figure 2). HmaL23–HmaL29DSP eluted between 180 and 220 mM KCl. By this prefractionation, approximately 60–70% of the contaminating protein could be removed. For further purification, the cross-link-containing fractions were pooled, desalted, and concentrated by RP-HPLC. Subsequent rechromatography by HPLC on Vydac C4 ultimately yielded the purified protein pair (Figure 3). HmaL23–HmaL29 eluted at 35% 2-propanol. Homogeneity was checked by SDS–PAGE and 2D-PAGE (Figures 3b and 4). A total of approximately 7 nmol of purified cross-link was isolated from 25 000 A_{260} of cross-linked 50S subunits.

The monomeric proteins of the protein pair were identified by 2D-PAGE after cleavage of the cross-link bridge by β-mercaptoethanol. Under the mild cleavage conditions, the protein pair was not cleaved completely. Figure 4 shows the electrophoretogram which clearly identifies the proteins HmaL23 and HmaL29 as the constituents of the cross-link. Additional confirmation of this result by N-terminal sequence analysis of the intact protein pair revealed that only HmaL29 was accessible to Edman degradation in the DSP as well as in the DEB cross-linked product. From the sequence of

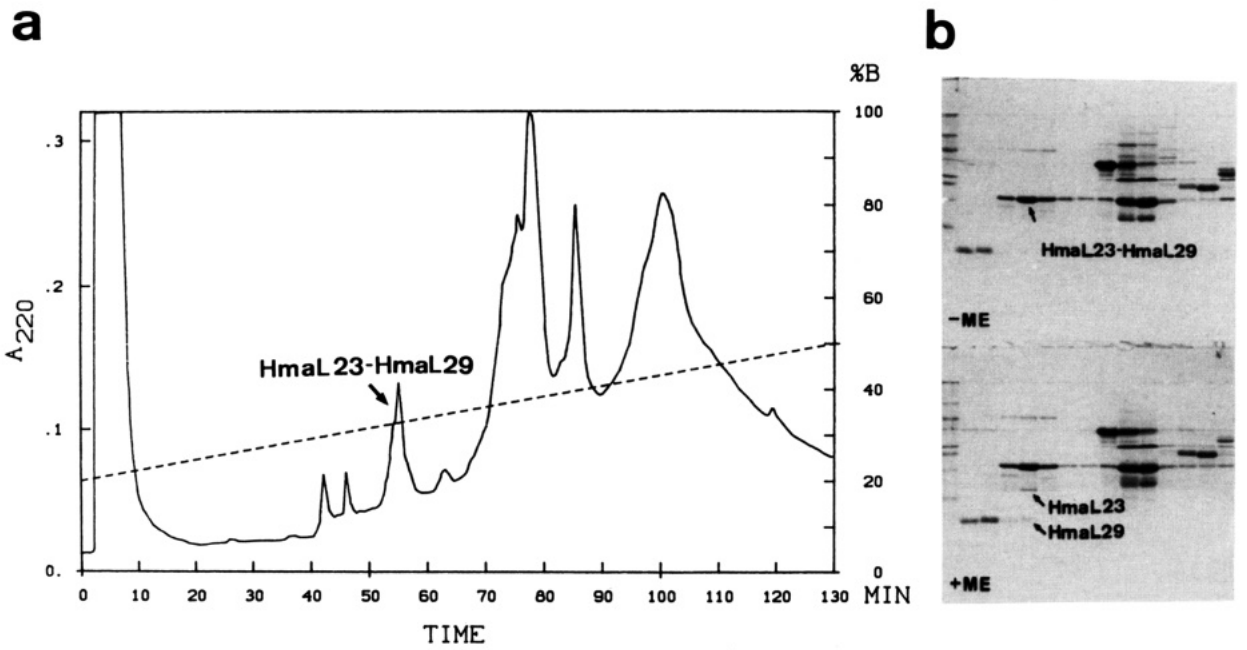

FIGURE 3: Isolation of HmaL23–HmaL29DSP by RP-HPLC. (a) Prefractionated HmaL23–HmaL29DSP was rechromatographed on Vydac C4 using a gradient of 0.1% aqueous TFA (buffer A) and 0.1% TFA in 2-propanol (buffer B): 20% B to 50% B in 130 min. The peak containing HmaL23–HmaL29DSP is indicated. (b) SDS–PAGE of HPLC fractions. The cross-link was identified without treatment (–ME) and after treatment of the protein with β-mercaptoethanol (+ME) prior to electrophoresis. The positions of the cross-linked protein pair and of the monomeric proteins are indicated.

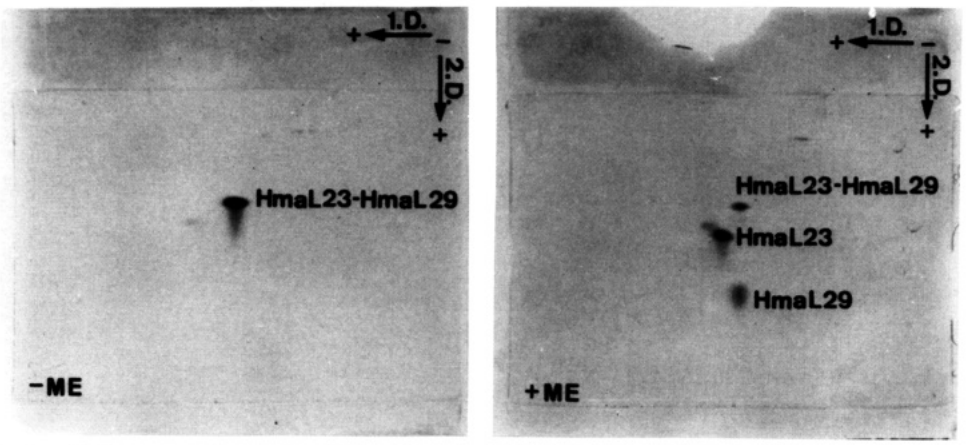

FIGURE 4: Identification of the monomeric proteins of HmaL23–HmaL29DSP. The purified cross-link was analyzed by 2D-PAGE prior to (–ME) and after (+ME) treatment with β-mercaptoethanol. The protein spots corresponding to HmaL23–HmaL29, HmaL23, and HmaL29 are indicated.

HmaL23, it was known that the N-terminal amino acid is not modified under native conditions (Hatakeyama & Kimura, 1988). Therefore, chemical blockage of the protein was assumed to result from reaction of the α-amino group with the cross-linking reagents (see below).

Identification of Cross-Linked Amino Acids in HmaL23–HmaL29DSP. Localization of the cross-linking site within the protein pair was achieved by enzymatic fragmentation of the intact cross-link and subsequent N-terminal sequencing of the resulting cross-linked peptide pair. For this purpose, lysylendoproteinase (LysC) was chosen because a limited number of larger peptides are expected, thereby facilitating the analysis of the peptide map. HmaL23–HmaL29DSP was digested in the presence of 4 M urea in order to dissolve the protein pair completely. The activity of the protease was not reduced under these conditions. Figure 5a shows the separation of the LysC peptides of HmaL23–HmaL29DSP by HPLC. The peptide bearing the cross-linking site was identified by treatment of the peptide mixture with β-mercaptoethanol prior to chromatography. The cross-linked peptide disappeared upon cleavage while another peptide with a shorter retention time appeared in the chromatogram (Figure 5b). The second peptide of the cross-link coeluted with another LysC peptide and could not be identified in the complex peptide map. The cross-linked peptide was subjected to N-terminal sequencing. Only one sequence was obtained, namely, the C-terminal peptide of protein HmaL29 spanning the region from Ala-52 to Glu-70. At the position of Lys-57 we could not identify the PTH derivative of lysine in the sequencing chromatogram, which clearly indicated that this amino acid must be involved in cross-link formation. Additionally, no other amino acid was present in this peptide region with reactivity toward DSP. Sequencing of the cleaved monomeric peptide resulted in the same HmaL29 sequence observed for the cross-linked complex, except that a modified PTH-lysine derivative was detected between PTH-tyrosine and PTH-arginine in the sequencing profile. This modification results from half of the DSP bridge which remains bound to lysine after reduction of the cross-link. Since no peptide from protein HmaL23 was found upon sequencing of the cross-linked

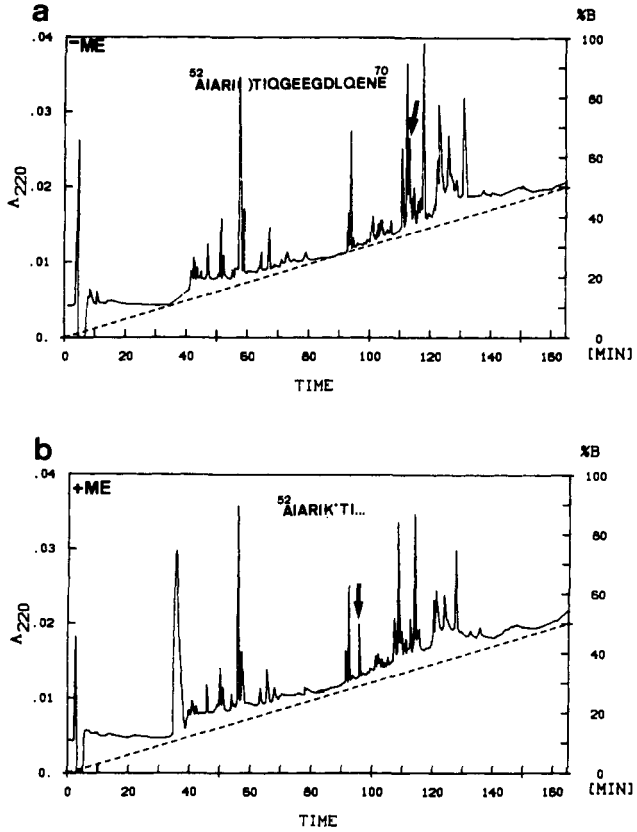

FIGURE 5: LysC peptide map of HmaL23–HmaL29DSP. The purified cross-link was digested with LysC, and the resulting fragments were separated by HPLC on LiChrospher C18 using a gradient of 0.1% aqueous TFA (buffer A) and 0.1% TFA in acetonitrile (buffer B): 0% B to 50% B in 165 min. Peptides were chromatographed either directly (–ME) (a) or after treatment with β-mercaptoethanol (+ME) (b). The positions of the cross-linked peptide and of the cleaved peptide are denoted by arrows. N-Terminal sequencing of the cross-link and of the monomer revealed only sequences from HmaL29, which are shown in the insert. K* symbolizes the modified PTH-lysine derivative obtained after cleavage of the cross-link.

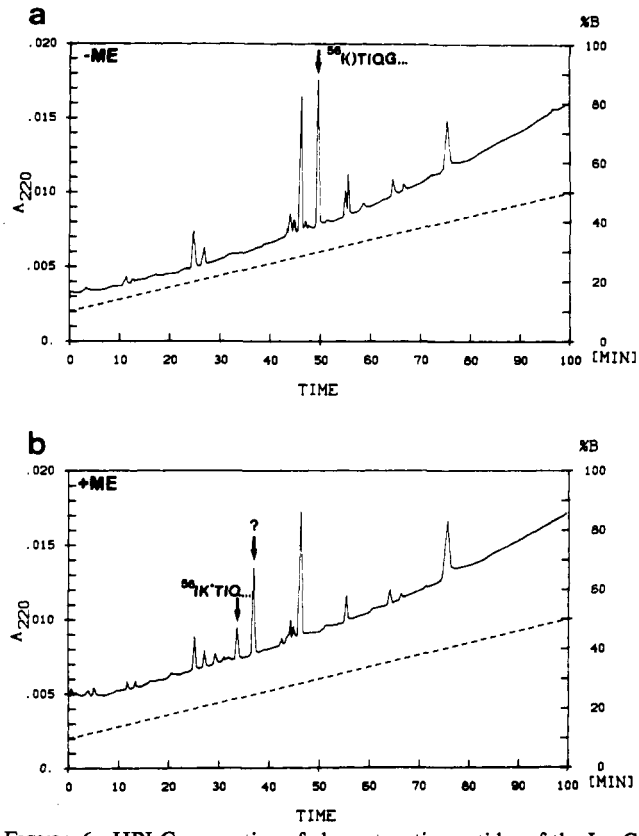

FIGURE 6: HPLC separation of chymotryptic peptides of the LysC fragment of HmaL23–HmaL29DSP. Peptides were analyzed under the same chromatographic conditions as in Figure 5. (a) The cross-linked peptide with the corresponding sequence is marked by the arrow. (b) The monomeric peptides are obtained after cleavage of the double peptide with β-mercaptoethanol and are marked by arrows. Only one peptide could be sequenced, whereas the other peptide was not accessible to Edman chemistry. K* symbolizes the modified PTH-lysine which was obtained after cleavage of the cross-linked peptide.

peptide, we conclude that the N-terminal region of this protein must be part of the cross-linking site. In order to determine the exact location within the N-terminal fragment of HmaL23, the original cross-linked LysC peptide was further digested with chymotrypsin to generate smaller sized peptides. Figure 6 shows the HPLC separation of the chymotryptic peptides prior to and after reduction with β-mercaptoethanol. The resulting monomeric peptides were clearly identified. N-Terminal sequence analysis of the intact peptide complex yielded the same cross-linked peptide as that obtained after LysC digestion, except that the C-terminal fragment of HmaL29 started at Ile-56. No further sequence from HmaL23 could be detected in the sequence chromatogram. Sequencing of the monomers revealed only the fragment from HmaL29, whereas the HmaL23 peptide remained chemically blocked and inaccessible to Edman degradation. Therefore, plasma desorption mass spectrometry was used for determination of the missing sequence information in the chymotryptic cross-linked fragment. The mass of the cross-linked peptide was determined to 2623.9 (not shown), which is in excellent agreement with the mass calculated from the sequenced fragment of HmaL29, Ile-56 to Glu-70, plus the mass of the DSP bridge (M_r 176) and the mass of the N-terminal fragment of HmaL23 spanning the region from Ser-1 to Lys-6. Hence, there is clear evidence that the α-amino group of Ser-1, not the ε-amino group of Lys-6, is involved in cross-link formation.

Final confirmation came from a cross-linked peptide which was obtained after cleavage of the chymotryptic peptide with thermolysin. Figure 7a shows the HPLC profile of the obtained fragments. All peptides were analyzed by mass spectrometry. One peptide gave a mass of 941.6, corresponding to the structure presented in Figure 7b. This fragment contained the HmaL29 peptide Ile_{56}-Lys-Thr and the HmaL23 N-terminal peptide Ser_1-Trp-Asp, including the DSP bridge between Lys-57 of HmaL29 and Ser-1 of HmaL23. From this result, we can unequivocally exclude that Lys-6 from HmaL23 participates in cross-link formation.

Identification of Cross-Linked Amino Acids in HmaL23–HmaL29DEB. For precise localization of the cross-linking site within the DEB protein pair, the same set of experiments described above was applied to HmaL23–HmaL29DEB. Starting with LysC digestion of the intact complex and HPLC separation of the resulting fragments, a cross-linked peptide was identified which yielded two shorter peptides after cleavage of the butanediol bridge with periodate (Figure 8a,b). The monomeric peptides are well resolved from the other digestion products. N-Terminal sequencing of the cross-linked double peptide resulted in the same C-terminal fragment of HmaL29 observed in the HmaL23–HmaL29DSP cross-link, spanning the region from Ala-52 to Glu-70 with a gap at Lys-57; no second sequence was found originating from protein HmaL23. Therefore, we assumed the participation of the N-terminus of HmaL23 in the cross-link formed by treatment with DEB. Final confirmation of this came from mass analysis of the

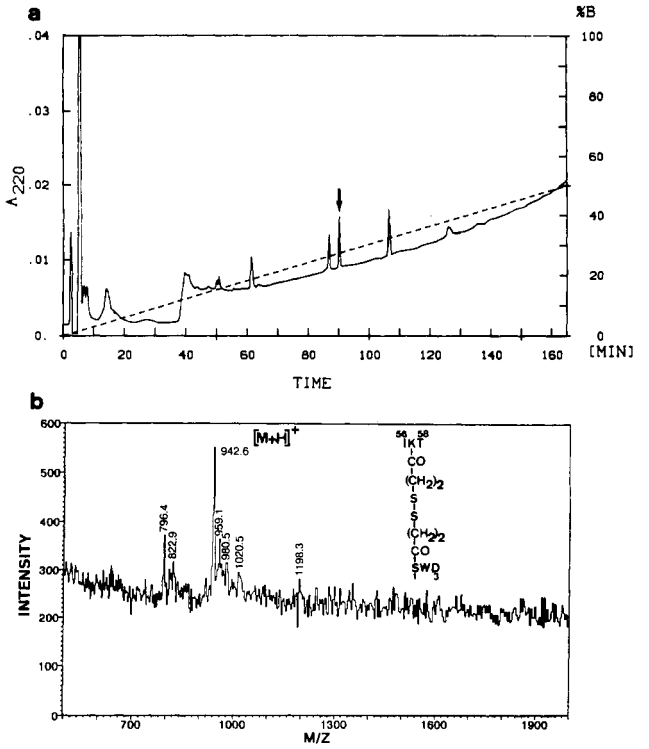

FIGURE 7: Identification of the cross-link site in HmaL23–HmaL29DSP. (a) The cross-linked chymotryptic peptide shown in Figure 6 was further digested with thermolysin, and the resulting fragments were separated by HPLC under the conditions shown in Figure 5. The cross-linked thermolysin peptide is indicated by the arrow. (b) Plasma desorption mass spectrogram of the smallest cross-linked peptide. The molecular ion $[M+H]^+$ with the mass of 942.6 corresponds exactly to the peptide structure (M_r 941) shown in the insert.

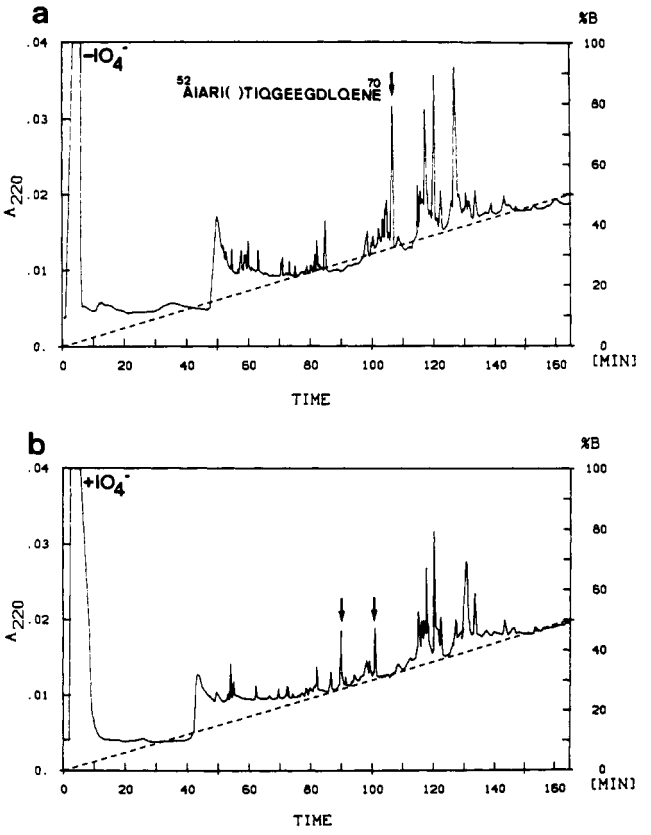

FIGURE 8: LysC peptide map of HmaL23–HmaL29DEB. The purified cross-link was digested with LysC, and the resulting fragments either were directly subjected to HPLC ($-IO_4^-$) (a) or were subjected after cleavage with periodate ($+IO_4^-$) (b). Chromatographic conditions were as in Figure 5. The original cross-linked peptide and the two monomeric peptides are marked by arrows. N-Terminal sequence analysis of the cross-linked peptide yielded the HmaL29 sequence shown in the insert with a gap at position 57.

cross-linked LysC peptide (Figure 9). The mass of 2947.1 corresponds exactly to that expected for the peptide of HmaL29 from Ala-52 to Glu-70 connected by a butanediol bridge via Lys-57 to Ser-1 of the HmaL23 peptide comprising residues Ser-1 to Lys-6. Due to chemical blockage of this latter peptide to N-terminal sequence analysis and the molecular mass of the cross-linked fragment, Lys-6 of HmaL23 was also unambiguously excluded from participation in the cross-linking reaction with DEB.

DISCUSSION

H. marismortui has been chosen as a representative of the archaebacteria to study the protein topography of this type of ribosome. This organism can be cultivated easily to yield sufficient amounts of ribosomes for cross-link site analysis at the amino acid level. So far, the complete primary structures of more than 40 ribosomal proteins of *H. marismortui* are known (Arndt et al., 1991). Furthermore, much progress has been made in the crystallization of ribosomal particles from this organism, and crystals which diffract to high resolution have been obtained (Makowski et al., 1987; von Böhlen et al., 1991). However, additional structural data and topographical information are necessary for correct interpretation of the complex X-ray diffraction pattern. Therefore, we investigated in situ protein neighborhoods within the 50S ribosomal subunit of this organism by chemical cross-linking. This approach enabled us to compare directly our results with cross-linking data from organisms incorporated in a protein model of the large ribosomal subunit from *E. coli* (Walleczek et al., 1988).

For this purpose, we chose the cleavable and homobifunctional cross-linking reagents DEB and DSP. These reagents yield stable cross-linking products in high yield under mild reaction conditions. Furthermore, their specificity and reactivity were not reduced in the high-salt environment necessary to retain the native structure of halophilic ribosomes. Cleavage of the cross-link bridge under mild conditions facilitated identification of the monomeric proteins during purification of the cross-linked complex.

In our study, HmaL23–HmaL29 was identified as the major protein pair formed both with DSP and with DEB. Purification of the two complexes was achieved by a two-step chromatography procedure. The usefulness of a combination of conventional ion-exchange chromatography and RP-HPLC has been proven already in cross-linking studies on eubacterial ribosomes (Pohl & Wittmann-Liebold, 1988; Herwig, 1990). The adaptation of the chromatographic conditions for the separation of proteins from *H. marismortui* resulted in the isolation of sufficient amounts of highly purified HmaL23–HmaL29. Identification of the constituents of the protein pair was performed by 2D-PAGE, as well as by N-terminal and internal sequence analysis, sequencing methods being the most reliable approach for unambiguous results. Due to alterations in the electrophoretic mobility of the chemically modified proteins, identification solely on the interpretation of patterns obtained by 2D-PAGE has often given inconsistent results. The determination of cross-links by immunochemical methods, such as applied in the analysis of complexes from *E. coli* (Stöffler et al., 1988), is independent of the electrophoretic migration but requires a complete set of highly specific antibodies.

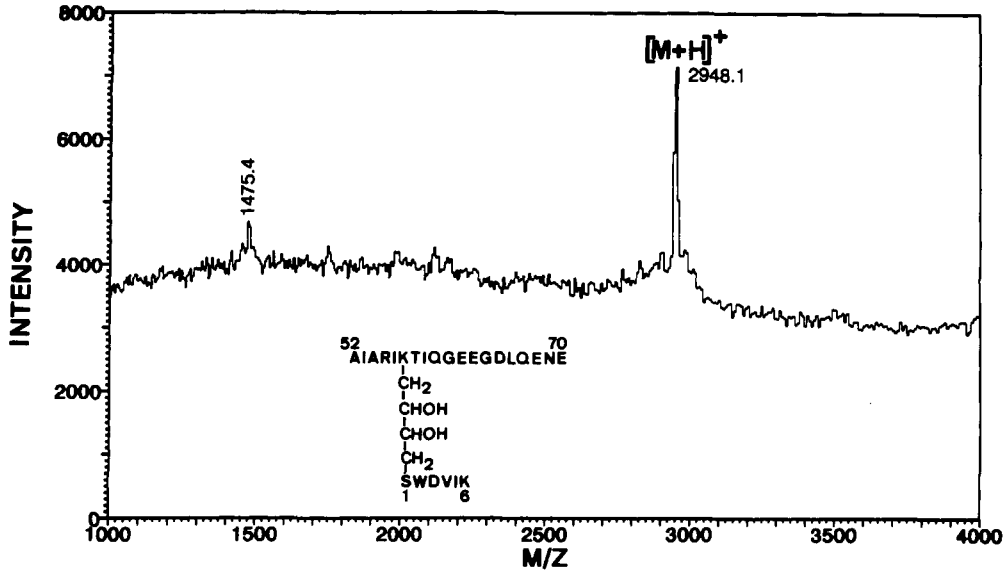

FIGURE 9: Identification of the cross-link site in HmaL23–HmaL29DEB. The cross-linked LysC peptide from Figure 8a was analyzed by plasma desorption mass spectrometry. The mass of 2947.7 (mass ion of 2948.1) corresponds exactly to the peptide structure shown in the insert. Lys-6 of HmaL23 is not part of the cross-link site because the peptide was inaccessible to Edman degradation.

HmaL23–HmaL29 is homologous to the protein pair L23–L29 of *E. coli* (Walleczek et al., 1988) and *B. stearothermophilus* (Brockmöller & Kamp, 1986). Cross-linking of homologous proteins in eubacteria and archaebacteria is reported here for the first time and indicates a conservation of this domain in ribosomes from diverse evolutionary kingdoms. However, in *E. coli* and in *B. stearothermophilus*, L23 and L29 were cross-linked exclusively by short cross-linking reagents, namely, *o*-PDM (5.2 Å) and DEB, respectively. No cross-linking was observed using DSP in *E. coli* 50S subunits.

In the DEB protein pair L23–L29 of *B. stearothermophilus*, the cross-link site has been determined at the amino acid level (S. Herwig, V. Kruft, K. Eckart, and B. Wittmann-Liebold, *J. Biol. Chem.*, in press). Using N-terminal sequence analysis and mass spectrometry, Lys-4 of BstL29 has been identified to cross-link to the α-amino group of Met-1 in BstL23. Hence, both in *H. marismortui* and in *B. stearothermophilus*, the N-terminal amino acid of L23 is involved in cross-link formation while the cross-linked amino acids in L29 lie within different regions of the proteins. Thus, we conclude that the fine structure within this ribosomal domain is at least partially conserved.

The finding that identical amino acids are involved in both the DSP- and DEB-induced cross-links was unexpected. The difference in length of the two reagent molecules is about 8 Å. Since the protein pair was formed in good yields and cross-linking was highly reproducible, we could exclude that our data result from partly denatured ribosomes. We conclude that either the cross-linking reagents and/or the N-terminal region of HmaL23 and the C-terminal peptide of HmaL29, respectively, must be extremely flexible. Secondary structure analysis based on the algorithm from Chou and Fasman (1974) predicts a β-turn motif within this area in HmaL23. Such secondary structure elements are known to be less rigid than α-helices or β-sheet motifs. Thus, in further cross-linking studies, the flexibility of peptide chains and reagent molecules should be considered when short distances between neighboring proteins are interpreted.

The cross-linking of Ser-1 from HmaL23 to Lys-57 to HmaL29 is interesting with respect to the position of protein L23 within the 50S subunit. Both L23 and L29 are susceptible to limited proteolysis of native ribosomes and can be located at least partially at the surface of the *E. coli* 50S subunit (Kruft & Wittmann-Liebold, 1991). Using immunoelectron microscopy, L23 has been localized in the vicinity of L29 at the base on the solvent side of the large subunit (Stöffler-Meilicke et al., 1983; Hackl & Stöffler-Meilicke, 1988). Further topographical data for an adjacent location of the two proteins on the 50S subunit come from protein–RNA cross-linking studies (Wower et al., 1981), which positioned L23 and L29 at neighboring helices in 23S RNA. On the other hand, photoaffinity labeling to the antibiotic puromycin (Jaynes et al., 1978; Weitzmann & Cooperman, 1985) and to the aminoacyl moiety of tRNA (Ofengand et al., 1986) favors a location of L23 in the proximity of the peptidyltransferase center, between L1 and the central protuberance on the interface side of the 50S subunit. These data are supported by cross-linking data from Traut and co-workers (Traut et al., 1986), who identified cross-links between L23 and proteins L15, L16, and L27. From our results and those from S. Herwig, V. Kruft, K. Eckart, and B. Wittmann-Liebold (*J. Biol. Chem.*, in press), we conclude that the N-terminal part of L23 is in the vicinity of L29. If we assume an elongated structure of L23 [previously demonstrated by physicochemical studies of Giri et al. (1984)], the C-terminal part of L23 could span the body of the 50S subunit and reach the peptidyltransferase center. Interestingly, this structure of L23 has been predicted by Nagano et al. (1988) and is in good agreement with our results.

As we can see from our results, protein–protein cross-linking is a valuable approach in the evaluation of the quaternary fine structure of archaebacterial ribosomes. However, further studies will be necessary to investigate more protein–protein neighborhoods and protein–RNA contact regions to compare the ribosomal structure of eubacteria and archaebacteria.

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