Preparation and Comparison of RP-HPLC of Crosslinked Ribosomal Protein Pairs by Chromatography on PLRP- and Vydac-Columns

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Summary
50S subunits of Bacillus stearothermophilus ribosomes were isolated and crosslinked with the homobifunctional reagent diepoxybutane. Two protein-protein crosslinks consisting of the proteins L23-L29 and L3-L19 could be purified on a preparative scale using conventional column chromatography followed by a combination of different HPLC techniques. This procedure allows the isolation of the crosslinks in amounts high enough for the determination of the crosslinked amino acids.

Introduction
Ribosomes are ubiquitous organelles and occur in all phylogenetic kingdoms. They are complexes of different RNA strains and various proteins of unique features. Their sizes and numbers in the different kingdoms vary considerably [1]. At present many ribosomal proteins deriving from eubacteria, archaeobacteria and eukaryotes have been sequenced either by protein-chemical methods or indirectly by DNA-sequencing of the genes [2]. Considerable progress has also been made in knowledge of the topographical arrangement of the ribosomal constituents. For this latter purpose a combination of different methods has been applied such as immune electron microscopy [3], neutron scattering [4, 5], 3D-image reconstruction [6], reconstitution experiments [7, 8], X-ray structure analysis [9] and crosslinking of neighbouring proteins within the particles [10-24]. Chemical crosslinking of proteins yields fine structure information since it permits the identification of neighbouring amino acids and consequently predictions about the spatial orientation of the proteins on the molecular level.

In this paper we show the purification and identification of two crosslinked protein pairs within the 50S subunits of Bacillus stearothermophilus ribosomes (L23-L29 and L3-L19) after incubation with the homobifunctional reagent diepoxybutane (DEB). This reagent had previously been employed as a protein-RNA crosslinking reagent in the Escherichia coli ribosome [25] and was found to be highly suitable for generating protein-protein crosslinks in relatively high yields in 30S ribosomal subunits of Escherichia coli [10, 12] and Bacillus stearothermophilus [26]. As DEB is soluble in water it allows crosslinking of proteins under physiological conditions, avoiding conformational changes of the ribosomes or its subunits during the crosslinking reaction. The resulting crosslink is stable during all protein purification steps, and the short length of the reagent (4 Å) guarantees specific reactions. At neutral pH, DEB reacts predominantly with the SH-groups of cysteine residues and with histidines, forming covalently bound carbon atom bridges [10, 11]. These contain vicinal hydroxyl-groups which are easily cleavable with sodium periodate. Under the mild reaction conditions used for crosslinking ribosomal proteins the yields of crosslinks are somewhat limited, and large scale isolations followed by sensitive HPLC-methods for further purification must be applied. Here, we compare HPLC reversed-phase separations on C4 and C18-coated columns with chromatography on polymer supports and preparation on ion-exchangers.

Experimental
Chemicals
Diepoxybutane was obtained from Serva (Heidelberg, FRG). Sodium(meta)periodate was purchased from Fluka (Buchs, Switzerland). 2-Propanol and acetonitrile were Lichrosolv grade from Merck (Darmstadt, FRG). Trifluoroacetic acid (Fluka) was distilled over CaSO4 · 0.5 H2O and redistilled over glass without additions. CM-Sepharose CL6B was obtained from Pharmacia (Heidelberg, FRG). All other chemicals were pro analysis grade from Merck.
Preparation of 50S Ribosomal Subunits and Crosslinking with Diepoxybutane

Growth of Bacillus steaothermophilus (strain 799), preparation of ribosomes and ribosomal subunits have been described earlier [26]. Crosslinking of 50S subunits with 1% (v/v) diepoxybutane was done exactly as described [26]. Total protein extracts were obtained by acetic acid extraction [27].

Periodate Cleavage

Purified crosslinked proteins were cleaved for 15 min in 100 μl 0.1% (v/v) TFA at pH 2 containing 10 mM freshly dissolved sodium(meta)periodate. Oxidation was stopped by direct injection of the released protein mixture into the HPLC column.

Preparative Isolation of Crosslinked Proteins by Ion-Exchange Chromatography

After acetic acid extraction the lyophilized protein extract was redissolved in equilibration buffer containing 10 mM phosphate, pH 7.6, 6 M urea, 10 mM methylamine and 6 mM β-mercaptoethanol and pre-fractionated by conventional ion-exchange chromatography on a CM-Sepharose CL6B column (30 cm x 5 cm). The proteins were eluted at ambient temperature using a gradient of 0 to 0.5 M KCl in equilibration buffer. The flow rate was 4 ml/min and 20 ml fractions were collected.

Desalting of the Protein Fractions

Minute amounts of proteins intended for analysis by 2D micro gel electrophoresis were desalted by precipitation on ice with 100 μl 0.2% (w/v) sodium desoxycholate and 200 μl 50% (v/v) trichloroacetic acid per ml sample volume.

Desalting and concentration of higher amounts of protein in dilute solution were carried out on reversed-phase columns (30 x 16 mm) equilibrated with aqueous 0.1% TFA using 100% 2-propanol as eluent [28].

Size Exclusion Chromatography

Size exclusion HPLC was performed on 300 x 21.5 mm Spherogel-TSK 3000 SW columns (purchased from Beckman, Palo Alto, CA, USA) in a buffer containing 0.1 M ammonium acetate, pH 4.1, and 6 mM mercaptoethanol. The HPLC system consisted of a HPLC pump, model 477A (Knauer, Berlin, FRG), a Rheodyne injection valve, no. 7100 (Rheodyne, Berkeley, CA, USA) and an UV/VIS filter photometer (Knauer). The flow rate was 1 ml/min and fractions of 1 ml were collected.

Separation of Crosslinked Proteins on Reversed Phase

For further purification the crosslinked 50S ribosomal proteins were separated on laboratory packed Vydac reversed phase columns (C4 and C18, particle size 5 μm, pore size 300 Å, 250 x 4.6 mm; The Separation Group, Hesperia, CA, USA) and on PLRP columns (particle size 10 μm, pore size 300 Å, 300 Å x 7.5 mm; Polymer Laboratories LTD., Shropshire, UK) at 40 °C using a volatile and UV-transparent buffer system made up of 0.1% TFA with 2-propanol or acetonitrile as eluents. The gradient was formed with two HPLC pumps (Knauer) equipped with an Apple controller (gradient program from LDC Milton Roy, Riviera Beach, Florida, USA). The sample was injected by a Rheodyne valve (Rheodyne, Berkeley, CA, USA) and detected at 230 nm in an UV/VIS filter photometer (Knauer).

Gel Electrophoresis

For the analysis of protein fractions one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [29]. Identification and check of purity was performed by two-dimensional micro gel electrophoresis according to the published method [30].

Microsequencing

N-terminal sequence determination was performed on an Applied Biosystems pulsed-liquid phase sequencer, model 477A, equipped with a model 120 PTH-AA analyser. Samples were dissolved in 100% TFA and applied to a TFA-treated polybrene-coated glass filter that had been precycled, as described previously [31].

Results and Discussion

50S subunits of Bacillus steaothermophilus ribosomes were treated with diepoxybutane under optimized crosslinking conditions [26]. Comparison of 2D-PAGE patterns of modified and unmodified subunits showed that three new spots became visible after crosslinking and disappeared after periodate cleavage (Figure 1). Previously, one of these complexes has been isolated and identified as the protein pair L23-L29 but the amount available was insufficient for further analysis on the amino acid level [26]. Another complex has been purified and identified as the crosslinked protein pair L3-L19 in this work. For both protein pairs a preparative purification procedure is presented. The third spot (signated as L3*) is probably an intramolecular crosslink of L3 [32].

Preparative Isolation of the Protein-Protein Crosslink L23-L29

The relatively low yield of the crosslinked complexes makes it necessary to start with a purification procedure that is applicable on a preparative scale. Figure 2a shows the elution profile of crosslinked 50S ribosomal proteins (50S-DEB) after ion exchange chromatography on CM-Sepharose CL6B. Small aliquots of each fraction were tested using one-dimensional SDS-PAGE for the presence of crosslinked proteins. The fractions were pooled, desalted by reversed phase chromatography and analysed by 2D micro gel electrophoresis (Figure 2b). L3*, L23-L29 and L3-L19 eluted successively in the range of 0.23-0.33 M KCl.

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