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Determination of Peptide Regions on the Surface of the Eubacterial and Archaebacterial Ribosome by Limited Proteolytic Digestion†

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Received August 5, 1991; Revised Manuscript Received September 26, 1991

ABSTRACT: Limited proteolysis was used in combination with two-dimensional gel electrophoresis, blotting, and amino acid sequence analysis to investigate the surface of intact ribosomal subunits at the peptide and amino acid level. Surface sites of 14 ribosomal proteins from *Escherichia coli* 50S subunits were determined using proteases with different specificities. To assess the evolutionary conservation of ribosomal topography among eubacteria, large subunits from *Bacillus stearothermophilus* were also subjected to limited proteolysis. The results obtained indicate a conservation of the three-dimensional ribosomal structure at the peptide level. The data for the eubacterial ribosomes are in full agreement with the model of the 50S protein topography derived from immunological data. Furthermore, peptide surface regions of archaebacterial ribosomes have been investigated. The results presented in this work prove that limited proteolysis can successfully be applied to halophilic and thermophilic ribosomes from archaebacteria.

Ribosomes are an essential part of the protein biosynthetic pathway in all living cells. Despite this unique function, ribosomes isolated from species of different kingdoms vary considerably in size and number of ribosomal components (Wittmann, 1986). To understand how structurally diverse ribosomes maintain a common function in the various organisms, the primary structures of the ribosomal components have to be determined, their locations in the ribosome have to be identified, and the ribosomal topography has to be resolved at the molecular level. Therefore, data about the surface regions and interactions of the ribosomal components within the ribosome at the amino acid/nucleotide level have to be obtained. To date, the primary structures of about 500 r-proteins[1] (RIB databank, Max-Planck-Institut für Molekulare Genetik) and of more than one thousand rRNAs are known; still, the complete set of primary structures is only known for the *Escherichia coli* ribosome (Wittmann-Liebold, 1986; Noller et al., 1986).

Several approaches have been employed to localize the positions of the ribosomal components within the ribosome.

† This publication is dedicated to the memory of Dr. H. G. Wittmann. His encouragement and support during our work is deeply appreciated.

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[1] Abbreviations: r-proteins, ribosomal proteins; rRNA, ribosomal RNA; Lys-C, endoproteinase Lys-C; Glu-C, endoproteinase Glu-C; Eco, *Escherichia coli*; Bst, *Bacillus stearothermophilus*; Hma, *Halobacterium marismortui* (*Haloarcula marismortui*); 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; IEM, immunoelectron microscopy.

0006-2960/91/0430-11781$02.50/0

Intra-RNA, RNA–protein, and protein–protein cross-linking studies have resulted in molecular models of both 23S rRNA and 16S rRNA of *E. coli* (Brimacombe et al., 1988, 1990) and have identified the positions of the r-proteins in the *E. coli* subunits (Schüler & Brimacombe, 1988; Walleczek et al., 1988). These sets of data agree reasonably well with the protein locations determined independently by immunoelectron microscopy (IEM) (Stöffler-Meilicke & Stöffler, 1990) and neutron scattering (Moore et al., 1986; Nowotny et al., 1986).

Nevertheless, topographical data at the peptide or amino acid level derived from cross-linking experiments are only available for some proteins, e.g., the protein pairs S5–S8 (Allen et al., 1979) and S13–S19 (Pohl & Wittmann-Liebold, 1988; Brockmöller & Kamp, 1988), and some of the protein–RNA cross-links [for example, Maly et al. (1980) and Ehresmann et al. (1976)].

Recently, immunological methods are applied to investigate the surface topography of the ribosome. Peptide surface epitopes can be identified by use of anti-peptide antisera either raised against peptides isolated after digestion of isolated proteins (Walleczek et al., 1990) or raised against peptides synthesized according to sequence stretches with high predicted surface probability (Herfurth and Wittmann-Liebold, unpublished results). Alternatively, the use of monoclonal antibodies necessitates the characterization of the antigenic epitopes recognized by the different monoclonal antibodies (Syu et al., 1990), but topographical data of some surface epitopes can be obtained (Nag et al., 1987; Olson et al., 1991). However, all these approaches yield data on the ribosomal topography only for a limited number of peptides.

Limited proteolysis is widely used to investigate the structure–function relationships in proteins [see Wilson (1991) for a review]. Yet only in some experiments has limited proteolytic digestion been used to probe ribosomal structure (Crichton & Wittmann, 1971, and references therein; Gudkov & Gongadze, 1984; Gudkov & Bubenenko, 1989).

In this paper we employ limited proteolysis of intact ribosomal 50S subunits to identify amino acids and peptide regions at the surface of the particle. Analysis of ribosomal cores by two-dimensional polyacrylamide gel electrophoresis followed by blotting and amino acid sequence analysis after limited proteolysis reveals primary cleavage sites on the surface of the ribosome, i.e., surface regions of the corresponding proteins as well as fragments of the proteins that stay anchored in the cores. This approach is fast and experimentally straightforward provided all amino acid sequences are available. It adds valuable information about the surface topography and the fine structure of the ribosome. By an independent approach, limited proteolysis verifies existing data on the ribosomal topography. This paper presents peptide surface regions of the large ribosomal subunits from *E. coli*, *Bacillus stearothermophilus*, and *Halobacterium marismortui*. The results are discussed with respect to the available immunological, biochemical, and phylogenetic data.

Materials and Methods

All chemicals were of p.A. grade or of higher quality, obtained from Merck. Endoproteinases Lys-C and Glu-C were purchased from Boehringer, thermolysin and chymotrypsin were from Serva, and TPCK-treated trypsin was from Worthington.

Preparation of Ribosomes. Cells of *E. coli* strain MRE600 and *B. stearothermophilus* strain 799 were grown and ribosomal subunits were purified by sucrose gradient purification as detailed by Brockmöller and Kamp (1986). *E. coli* tight couple ribosomes were obtained following the procedure described by Rheinberger et al. (1988). Cells of the archaebacteria *H. marismortui* and *Sulfolobus acidocaldarius* (DSM 1616) were grown and large ribosomal subunits were prepared as described by Shevack et al. (1985) and Grote et al. (1986), respectively.

Limited Proteolysis of Intact Ribosomes and Ribosomal Subunits. Aliquots of 100 A_{260} of 50S or 70S ribosomes were subjected to limited proteolysis in 2 mL of the following buffers: TMA-I (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 30 mM NH_4Cl) was used for the eubacterial ribosomes, TMA-S (10 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 10 mM NH_4Cl, 100 mM KCl) was for ribosomes isolated from *S. acidocaldarius*, and TMA-H (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 500 mM NH_4Cl, 3 M KCl) was for halophilic ribosomes.

Proteolysis was carried out overnight at 37 °C at the following enzyme:substrate ratios: Lys-C was used at 0.01 unit/A_{260} ribosomes, and Glu-C, chymotrypsin, and trypsin were used at 1:100 (w/w). Ribosomes isolated from the thermophilic organisms were additionally subjected to limited proteolysis by thermolysin at 55 °C and an enzyme:substrate ratio of 1:100 (w/w). The reactions were stopped by 40-fold concentration of the cores in Amicon-10 concentrators followed by acetic acid extraction of the cores according to Hardy et al. (1969). Control experiments in which the cores were separated from released peptides by centrifugation through a cushion of 1 M sucrose yielded identical results (data not shown).

Two-Dimensional Polyacrylamide Gel Electrophoresis, Protein Blotting, and Amino Acid Sequencing. Two-dimensional polyacrylamide gel electrophoresis was carried out as described by Geyl et al. (1981) for proteins and fragments from cores of *E. coli*, *B. stearothermophilus*, and *S. acidocaldarius*; the first dimension gel system for the acidic proteins extracted from *H. marismortui* was according to Strøm and Visentin (1973), and the second dimension was according to Laemmli (1970). Protein blotting onto PVDF membrane (Millipore) for microsequencing was carried out following the procedures described by Walsh et al. (1988) and Choli and Wittmann-Liebold (1990). Protein fragments were sequenced directly in a Model 477A pulsed-liquid gas-phase sequencer equipped with a Model 120A PTH-amino acid analyzer (Applied Biosystems). Sequences were identified by comparison with sequences in the RIBO databank (Max-Planck-Institut für Molekulare Genetik).

Results

The 50S ribosomes from the Gram-negative eubacterium *E. coli* strain MRE600, the Gram-positive eubacterium *B. stearothermophilus*, and the archaebacteria *S. acidocaldarius* (DSM 1616) and *H. marismortui* were digested with various proteases as detailed under Materials and Methods.

Figure 1 shows examples of analyses by two-dimensional polyacrylamide gel electrophoresis of proteins extracted from intact 50S subunits and of the cores after limited proteolysis containing intact proteins and fragments thereof (indicated by arrows). The digestion conditions were optimized to leave the gross protein composition intact while generating a number of protein fragments. The cores were separated from released peptides by centrifugation and concentration in Amicon concentrators before acetic acid extraction of the proteins and large-sized fragments. Therefore, the new spots in the two-dimensional pattern must represent large peptides that were generated during digestion but stayed firmly anchored in the ribosomal core.

Figure 2 indicates the positions in the two-dimensional map

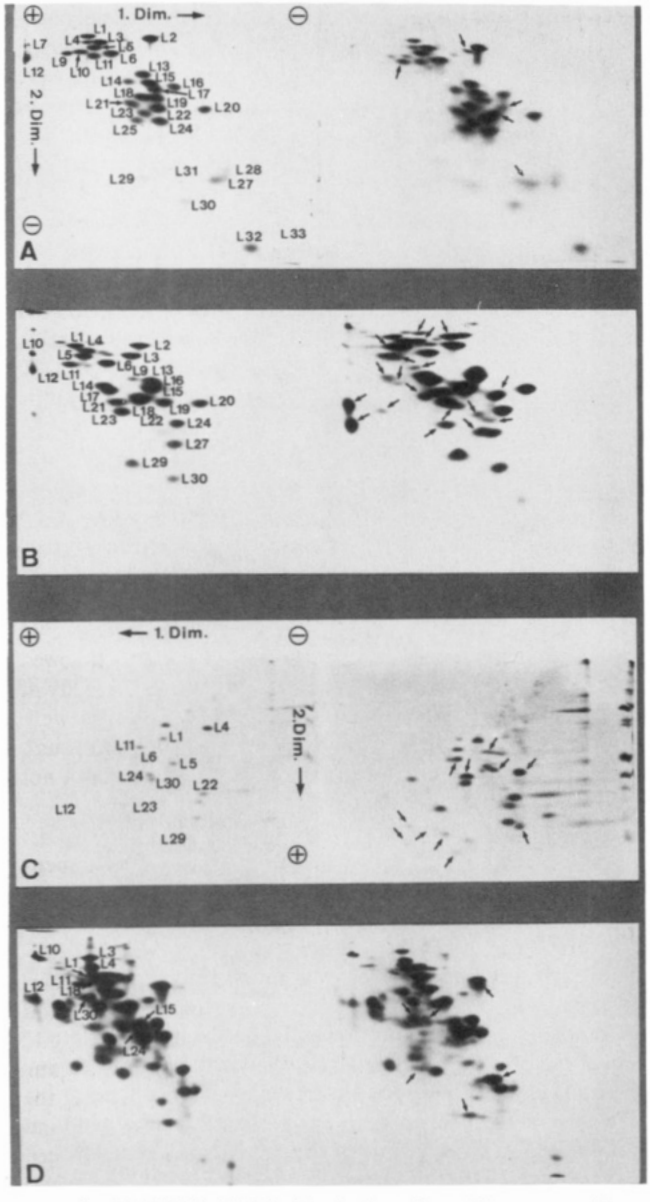

FIGURE 1: 2D PAGE of 50S subunits from four different organisms. In each, the left gel shows the control and the right gel shows the cores remaining after limited proteolysis. Proteins are numbered according to their homology to *E. coli* proteins; fragments generated by proteolysis are indicated by arrows. (A) *E. coli* 50S subunits digested with Lys-C; (B) *B. stearothermophilus* 50S subunits digested with chymotrypsin (electrophoretic directions as indicated for *E. coli*); (C) *H. marismortui* 50S subunits digested with Glu-C; (D) *S. acidocaldarius* 50S subunits digested with trypsin (electrophoretic directions as indicated for *E. coli*).

and the identification by sequence analysis of protein fragments generated during limited proteolysis of *E. coli* 50S subunits by three different enzymes (Lys-C, Glu-C, and chymotrypsin). Fragments that were generated in low yields that are not visible on the gel photographs were detected on slightly overloaded gels used for blotting and sequencing. Surface regions of the proteins in the intact subunits were directly identified when the protein spots yielded internal sequences that start immediately after a cleavage site. When spots yielded N-terminal sequences, the approximate lengths and net charges of the fragments were calculated from known sizes and net charges of neighboring proteins in the two-dimensional map. The cleavage site of the protein concerned could unambiguously be determined using the calculated charges and sizes of the fragments in combination with their primary structures and the specificities of the proteases employed. In each case the appearance of several fragments from one protein in a single digest (e.g., EcoL9 in Figure 2B) or in different digests (e.g., EcoL2 in Figure 2B,D; EcoL19 in Figure 2B,D) confirmed the deduced sites of surface exposure.

Figure 3 summarizes the results for deduced and directly determined surface sites after different digestions of the 50S ribosomal subunits of three different organisms, *E. coli*, *B. stearothermophilus*, and *H. marismortui*. Directly determined and deduced cleavage sites are indicated by arrows underneath the proteins. The enzymes used in this study retained their expected specificities. Depending on the sequence obtained (internal or N-terminal, as indicated by an arrow above the protein), the protein fragments proven to be anchored in the core are shown as thick bars. Bars of intermediate thickness mark extended surface-exposed peptide stretches with several cleavage sites.

For all ribosomes investigated, cleavage sites, and hence surface-exposed peptides, were identified in N-terminal, C-terminal, and internal regions of the cleaved proteins. Cleavage sites by digestion with Lys-C, Glu-C, and chymotrypsin analyzed on the 50S subunit from *E. coli* proved N-terminal surface exposure for proteins EcoL27 and EcoL29, C-terminal surface exposure for proteins EcoL2, EcoL10, EcoL14, EcoL17, EcoL19, and EcoL23, and exposure of internal regions for proteins EcoL1, EcoL4, EcoL5, EcoL9, EcoL11, and EcoL15 (Figure 3A). Treatment of large subunits of *B. stearothermophilus* with the same enzymes identified surface domains in regions which were also found in the *E. coli* counterparts for proteins BstL2, BstL5, BstL11, BstL19, and BstL23 (Figure 3B). Additionally, internal cleavage was observed for protein BstL6, and N-terminal exposure was shown for protein BstL2.

Analysis of protein fragments generated by limited proteolysis with Lys-C, Glu-C, chymotrypsin, and trypsin on 50S subunits from *H. marismortui* identified surface-exposed regions of proteins that have counterparts in the eubacterial kingdom as well as of proteins with no significant similarity to eubacterial ribosomal proteins (Figure 3C). Proteins HmaL1, HmaL2, and HmaL3 have C-terminal peptide regions exposed on the ribosomal surface; HmaL4 and HmaL5 have N-terminal regions exposed. Additionally, internal domains of HmaL3 and HmaL4 are accessible to proteolytic attack. Of the proteins found to be cleaved that have no significant homology to any eubacterial ribosomal protein, HL24, HL29, and HL5 [for nomenclature of archaebacterial ribosomal proteins, see Wittmann-Liebold et al. (1990)] have, respectively, C-terminal, N-terminal, and internal peptide domains exposed on the surface (Figure 3C).

To assess the protection of the interface side of the isolated 50S subunit by association with the small ribosomal subunit within the 70S particle, limited proteolysis was also carried out on tight couple ribosomes from *E. coli*. Figure 4 shows a two-dimensional pattern of the cores after digestion with Lys-C. Comparison with the patterns given in Figure 1A and 2A shows that the interface proteins EcoL2 and EcoL19 (Stöffler-Meilicke & Stöffler, 1990) are protected from cleavage in 70S ribosomes.

Discussion

A new approach to assign exposed peptide regions within intact ribosomes and ribosomal subunits has been developed employing limited proteolysis in combination with blotting from two-dimensional polacrylamide gels and direct microsequencing of the generated protein fragments. It allows the identification of cleavable peptide stretches on the surface of the ribosome or its subunits as well as the identification of

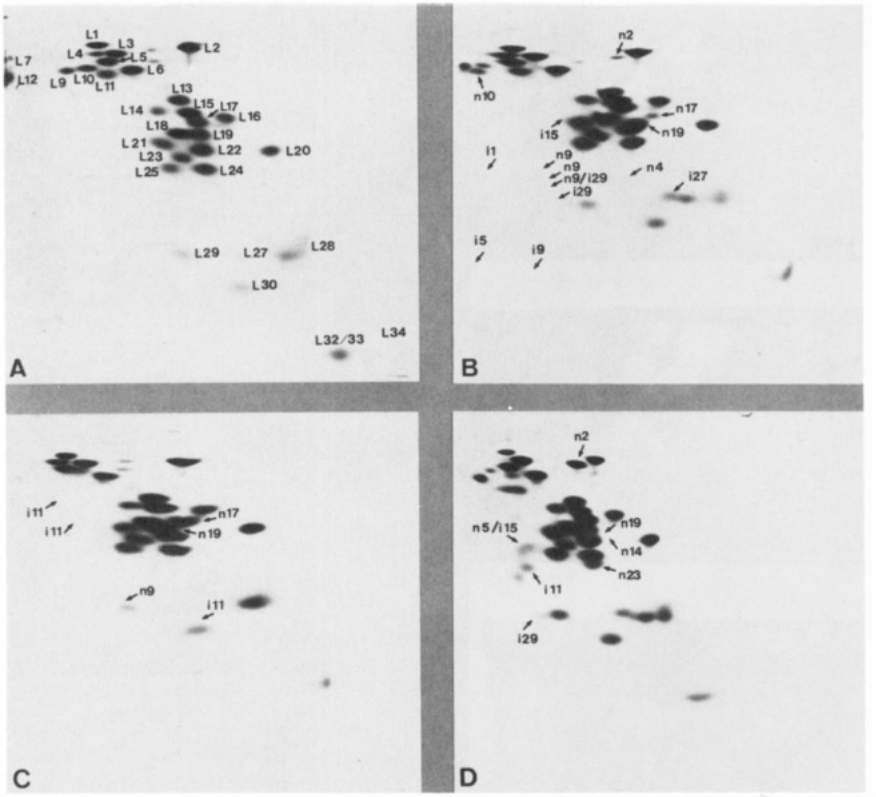

FIGURE 2: 2D PAGE of *E. coli* 50S cores. First dimension: left to right (plus to minus). Second dimension: top to bottom (plus to minus). Positions of core fragments generated by limited proteolysis are indicated by arrows. Numbering of the arrows gives the identities of the proteins obtained by amino acid sequencing. N-terminal or internal sequences obtained from the proteins are indicated by the prefix n or i, respectively. (A) control; (B) Lys-C digest; (C) Glu-C digest; (D) digest with chymotrypsin. Fragments generated in low yields may not be visible in this figure but were obtained by blotting and sequencing done from slightly overloaded gels.

peptide fragments that remain anchored in the core after proteolytic treatment. The method verifies the information about ribosomal surface topography obtained by other approaches, and it probes surface exposure of ribosomal proteins at the amino acid level.

Peptide regions and cleavage sites at the surface of the intact *E. coli* 50S subunit were determined for 14 proteins (Figure 3A). Those proteins have also been positioned in the subunit by IEM (Stöffler-Meilicke & Stöffler, 1990). Furthermore, proteins that were determined by us to have extended surface domains, e.g., proteins EcoL9, EcoL11, and EcoL15, have been reported to be connected by pairs of IgG molecules in dimeric immunocomplexes (Stöffler–Meilicke et al., 1983). Two reasons explain why peptide surface areas could not be determined for all proteins mapped on the ribosome by immunological methods. First, not all proteins exposing antigenic epitopes are necessarily accessible to the proteases chosen; and second, analysis of the supernatants after digestion by reversed-phase high-performance liquid chromatography and amino acid sequencing demonstrated that in *E. coli* the stalk proteins EcoL7/EcoL12, as well as some small proteins such as EcoL31, were completely digested in our experiments (data not shown).

Whereas exposed proteins yielded large fragments, proteins that are fully protected in the inner part of the particle were not affected under the limited proteolysis conditions employed here. Among those are the proteins that have so far not been positioned by the IEM approach, e.g., proteins EcoL3, EcoL13, EcoL16, EcoL21, and EcoL22 (Stöffler-Meilicke & Stöffler, 1990). Proteins EcoL2 and EcoL14, which could not be localized by IEM using antisera raised against the proteins from *E. coli* but were positioned in the 50S ribosome from *B. stearothermophilus*, were shown to have surface epitopes in this study using limited proteolysis on 50S ribosomes from *E. coli*.

Comparison of the data presented here with the assembly map of the 50S subunit from *E. coli* according to Herold and Nierhaus (1987) shows good correlation. Few of the proteins that are essential for in vitro early assembly ($RI^*_{50}(1)$ formation) or are constituents of the 4.0 M LiCl core (Dohme & Nierhaus, 1976; Spillmann & Nierhaus, 1978) are accessible to proteolytic attack. Those that are, e.g., proteins EcoL2, EcoL17, and EcoL29, are shown here to have distinct surface domains in the different digests. Conversely, all proteins that have extended surface domains (Figure 3A) are late assembly proteins, e.g., proteins EcoL9, EcoL11, and EcoL15.

Of the 5S rRNA binding proteins EcoL5, EcoL18, and EcoL25, which together with 5S and 23S rRNA form the central protuberance of the subunit, only protein L5 is accessible to protease in our experiments, whereas L18 and L25 seem to be completely protected. This finding is in agreement with the report by Christiansen and Garrett (1986) that even in an isolated complex of L18 with 5S rRNA the protein can only be cleaved under harsh tryptic treatment. Contrary to the stability of the central protuberance, a fragment of EcoL1, as well as numerous fragments of both EcoL9 and EcoL15, which form the L1 protuberance, were isolated. The stalk protuberance completely dissociates from the *E. coli* ribosome during proteolytic treatment. The nearly complete cleavage of EcoL10 indicates that the C-terminus of EcoL10 is surface exposed after dissociation of the stalk. This finding agrees with the report by Gudkov et al. (1980) that the C-terminus of EcoL10 interacts with the N-termini of the L7/L12 dimers (van Agthoven et al., 1975; Koteliansky et al., 1978).

Figure 5 shows a model of the topography of the proteins in the 50S subunit as determined by protein–protein cross-

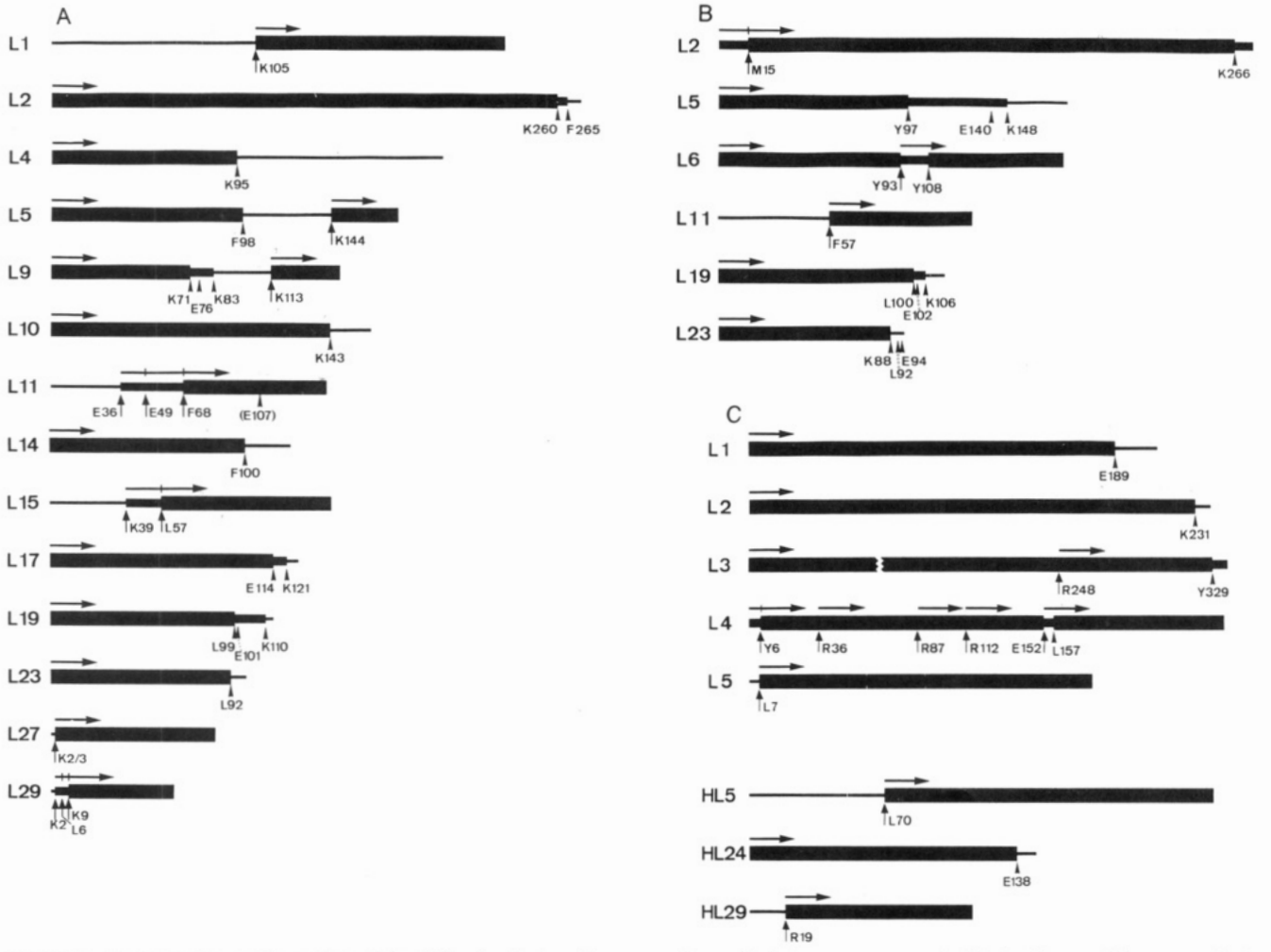

FIGURE 3: Cleavage sites on the surface of the 50S subunits from three organisms. Protein sequences are depicted as bars and drawn to relative size: (A) *E. coli*; (B) *B. stearothermophilus*; (C) *H. marismortui*. Forward right arrows indicate sequences obtained by direct sequencing. Cleavage sites are shown underneath the bars: arrows indicate directly determined cleavage sites and pointers indicate deduced cleavage sites. Thick bars mark sequence stretches that are anchored in the core after digestion; bars of intermediate thickness mark extended surface-exposed peptide stretches with several cleavage sites. Letters underneath the bars identify the amino acids at the cleavage sites (K, cleavage by Lys-C; E, cleavage by Glu-C; F, Y, L, or M, cleavage by chymotrypsin; R, cleavage by trypsin) and are numbered according to their positions in the primary structure.

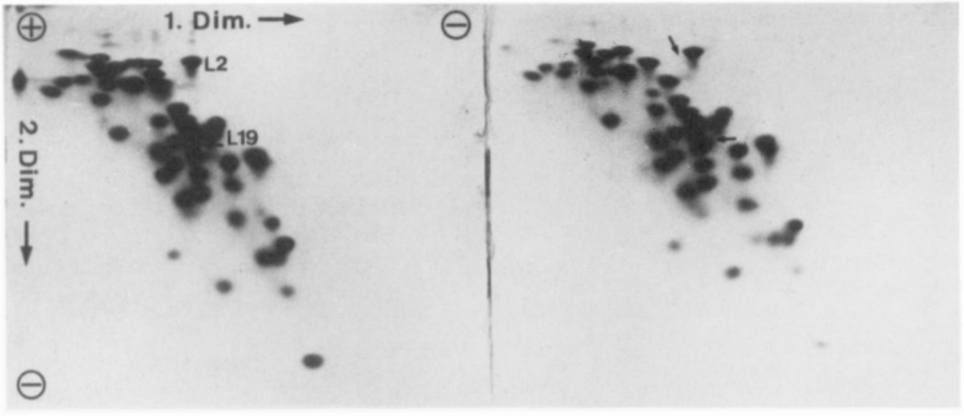

FIGURE 4: Limited proteolysis of *E. coli* tight couple ribosomes: (left) control; (right) digest with Lys-C. The positions of the fragments of EcoL2 and EcoL19 not generated in comparison to 50S subunits (Figure 1A) are indicated by arrows.

linking and IEM (Walleczek et al., 1988). Proteins shown in our experiments to have cleavable surface regions on the intact ribosome are depicted as dark spheres.

Limited proteolysis can further be employed to identify interface proteins and their surface peptide structures by comparing the data obtained from proteolytic treatment of 50S and 30S subunits with those from digestion of tight couple 70S ribosomes. Comparison of Figure 4 with Figure 1A shows that association of 50S and 30S subunits protects proteins EcoL2 and EcoL19 from digestion with Lys-C. This result also demonstrates that the cleavage sites deduced for proteins EcoL2 and EcoL19 after limited proteolysis overlap with the domains recognized by the antibodies in IEM.

We applied limited proteolysis to 50S ribosomal subunits isolated from different organisms. Data obtained by analysis of protein–protein cross-links at the amino acid level or IEM

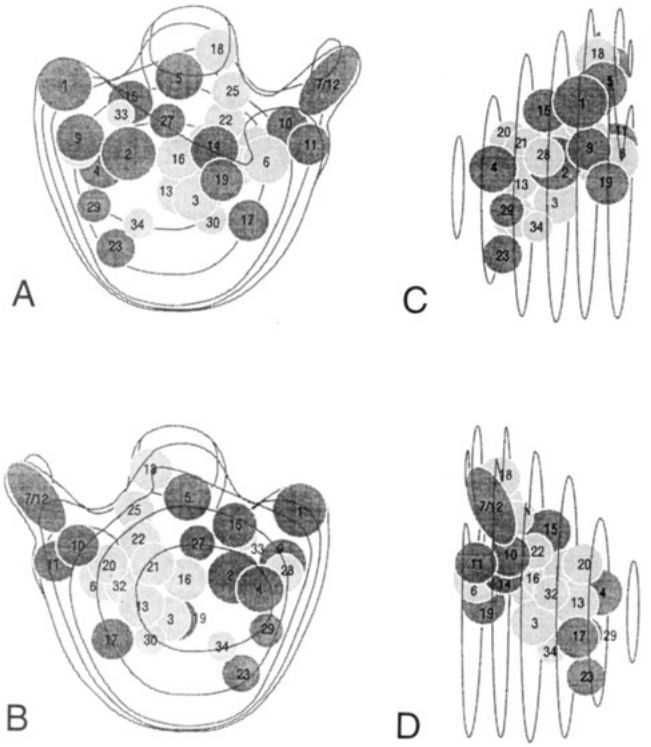

FIGURE 5: Model of the protein topography of the *E. coli* 50S ribosomal subunit [modified from Walleczek et al. (1988)]. Dark spheres denote proteins susceptible to proteolytic attack (this work).

on ribosomes of different origin (Pohl & Wittmann-Liebold, 1988; Brockmöller & Kamp, 1988; Stöffler-Meilicke & Stöffler, 1990) indicate an overall conserved domain structure at least between Gram-negative and Gram-positive eubacteria.

The cleavage sites detected on the 50S subunit from *B. stearothermophilus* prove that proteins BstL2, BstL5, BstL11, BstL19, and BstL23 are cleaved at corresponding sites compared to those from *E. coli*, indicating that for those proteins the surface domains are indeed conserved (Figure 3B). The detection of an additional cleavage site close to the N-terminus of BstL2 confirms the data obtained by Olson et al. (1991) and further narrows the exposed peptide areas to only the very N- and C-terminal domains. Again, the example of L2 demonstrates that primary cleavage sites for the proteases can also be domains accessible to antibodies.

The situation is less clear when ribosomes from eubacteria are compared with those from archaebacteria, since very little biochemical or topographical data on ribosomes derived from archaebacteria are known. An investigation of the surface structure of archaebacterial ribosomes at the peptide level is necessary because high quality ribosomal crystals have been obtained for the 50S subunit from *H. marismortui* (Makowski et al., 1987; von Böhlen et al., 1991). We started a detailed study to clarify whether eubacterial and archaebacterial ribosomes share common domains and to what extent topographical data derived from eubacteria can be transferred to predict structure–function relationships in archaebacterial ribosomes. Limited proteolysis can be performed on archaebacterial ribosomes, and the proteases do not lose their activity and specificity in the buffers necessary to perform limited proteolysis under native conditions (Figure 1C,D). For *H. marismortui*, the cleavage sites were directly determined or deduced analogous to the data obtained for the eubacterial ribosomes, taking into account the different gel system (Figure 3C). Cleavage sites on five proteins whose sequences are similar to eubacterial proteins were determined. HmaL2, the equivalent to EcoL2 and BstL2, is cleaved close to the C-terminus as was also shown for *E. coli* and *B. stearothermophilus*. HmaL3 is cleaved, whereas no cleavage sites were found for the eubacterial L3 proteins. The cleavage sites on the archaebacterial proteins HmaL1 and HmaL5 are in different positions compared to the sites shown in Figure 3A,B. Six fragments of protein HmaL4 were detected. Most of those cleavage sites are in positions neighboring inserted peptide regions compared to EcoL4, which is considerably shorter than HmaL4 (data not shown). This argues for the hypothesis that extensions on ribosomal proteins compared to shorter homologous proteins are likely to be found on the outside of the ribosome. The cleavage of HmaL3 illustrates this point further, since the C-terminus is an extension compared to EcoL3.

Three proteins with no known counterpart in the eubacterial ribosome were cleaved, namely HL5, HL24, and HL29. Of those, HL5, which is homologous to the eukaryotic protein L32 (Scholzen & Arndt, 1991), is especially interesting. The protein isolated from *H. marismortui* has an N-terminal extension of about 100 amino acids that is cleaved during proteolytic treatment and hence is exposed on the surface. In *S. acidocaldarius*, the extension is not present (A. T. Matheson, personal communication) and only the N-terminal region is accessible to protease (Kruft & Wittmann-Liebold, unpublished results).

In comparison to the surface regions determined for *E. coli* and *B. stearothermophilus*, both differences and similarities in the surface domain structure in *H. marismortui* 50S ribosomes have been detected. More data have to be accumulated for a detailed analysis of the surface domains of this ribosome.

Limited proteolysis on intact 50S ribosomes in combination with a molecular analysis of the cleavage sites has yielded data about surface regions at the amino acid level that are in complete agreement with all topographical data obtained by other approaches. The level of conservation of surface domain structures between archaebacteria and eubacteria can be evaluated in further studies when more sequence information is available from archaebacterial ribosomes. Limited proteolysis as employed here to map the ribosomal surface will help to refine the present model of the *E. coli* ribosome at higher resolution. Such a model is necessary to understand the structure–function relationships governing ribosomal function at the molecular level and will help to assign peptide backbones to areas of electron density when X-ray diffraction data of ribosomal crystals are available.

ACKNOWLEDGMENTS

We thank U. Kapp and H. Gaenge for technical assistance. *E. coli* tight couple ribosomes were a gift from Dr. K. H. Nierhaus, and large subunits from *S. acidocaldarius* were a gift from P. Henning (Max-Planck-Institut fur Molekulare Genetik). Dr. A. R. Subramanian is acknowledged for critical reading of the manuscript.

Registry No. Lys-C, 72561-05-8; Glu-C, 37259-58-8; trypsin, 9002-07-7; chymotrypsin, 9004-07-3; thermolysin, 9073-78-3; proteinase, 9001-92-7.

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