Characterization of Braun's Lipoprotein and Determination of its Attachment Sites to Peptidoglycan by ^{252}Cf-PD and MALDI Time-of-Flight Mass Spectrometry

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A strategy for the characterization of bacterial lipoprotein in this case Braun's lipoprotein (an outer membrane 7-ku lipoprotein) isolated from *Escherichia coli*—is described by time-of-flight mass spectrometric (TOF/MS) techniques [^{252}Cf plasma desorption (PD) TOF/MS and matrix-assisted laser desorption–ionization (MALDI) TOF/MS]. Covalent linkage of lipid at the N-terminal cysteine (posttranslationally modified to a *S*-[2,3-bis(acyloxy)-propyl]-*N*-acylcysteine) and, therefore, strict insolubility in aqueous solution constitute common features for this class of proteins. Relative molecular mass determination of the major molecular species of Braun's lipoprotein was obtained by selection of an appropriate mixture of organic solvents compatible with matrix/support materials useful for the mass spectrometric techniques applied. Minor components of this lipoprotein that differ only in the fatty acid composition of the lipid anchor were detected by PD TOF/MS after enzymatic release of the extremely hydrophobic N-terminal amino acid followed by selective extraction with chloroform. Part of the primary sequence of this lipoprotein was confirmed based on peptide fragment ions observed in the positive ion PD mass spectra of cyanogen bromide-generated peptide fragments that had been isolated previously by reverse phase high-performance liquid chromatography (HPLC). Peptidoglycan fragments that represent the attachment sites of lipoprotein to peptidoglycan were enzymatically released, separated by reverse phase HPLC, and finally characterized by time-of-flight mass spectrometric techniques (^{252}Cf-PD TOF/MS, MALDI TOF/MS). The results obtained with both techniques differed only in the better sensitivity obtained with MALDI TOF/MS, which consumed a factor of 100 to 1000 less material than with PD TOF/MS. *(J Am Soc Mass Spectrom 1995, 6, 892–905)*

Bacterial lipoproteins constitute a class of strongly hydrophobic, water-insoluble membrane proteins, whose N-terminal amino acid cysteine is posttranslationally modified to a *S*-[2,3-bis(acyloxy)-propyl]-*N*-acylcysteine residue [1]. These membrane proteins occur in the free form as well as covalently linked to the peptidoglycan layer as commonly observed among Gram-negative *Enterobacteriaceae*. Peptidoglycan itself represents a giant macromolecule that consists of long glycan strands of alternating 1β → 4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units with short peptide side chains (that typically contain 2–5 amino acids) linked to the MurNAc residue and that terminate with a 1,6-anhydro-MurNAc residue [2]. The complete three-dimensional shape of peptidoglycan is provided by cross-linkage of peptide side chains of different glycan strands to form a water-insoluble network surrounding the cytoplasmic membrane. When peptidoglycan is digested with a muramidase (e.g., lysozyme, *Chalaropsis* muramidase, etc.) to obtain

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water-soluble peptidoglycan fragments, the carbohydrate copolymer is cleaved into disaccharide units that retain their peptide side chains. Where a peptide side chain is not cross-linked to another peptide side chain, the resulting digest product corresponds to a disaccharide peptide and is therefore termed peptidoglycan monomer (e.g., a disaccharide tripeptide will then be called "Tri"). If such a monomer building block is cross-linked via a peptide bond to another monomer, the resulting muramidase digest product would be a bis-disaccharide peptide and, therefore, called peptidoglycan dimer. Examples of chemical structures of peptidoglycan monomers, dimers, and one trimer that occurs in muramidase digests of the peptidoglycan of *Escherichia coli* are given in Figure 1.

The bound form of such lipoproteins from *Enterobacteriaceae* is usually linked to the diamino acid (i.e., diaminopimelic acid) of disaccharide tripeptide units (compare compound 2 in Figure 1). Among other Gram-negative and also Gram-positive bacteria, lipoproteins have been detected only in the free form that is not covalently linked to any other biopolymer [1]. The biological functions and properties of these lipoproteins include maintenance of the outer membrane structure, nutrient transport systems, and a wide variety of different enzymatic activities as well as several different immunological reactions in animals and human beings [1]. A growing interest in pharmaceutical research for vaccines against bacterial infections is due to these immunological properties; thus, for example, there is an interest in the outer surface protein A (OspA) from *Borrelia burgdorferi*, a bacterium that is known to cause Lyme disease. Recently, lipid-linked OspA (but not the lipid-free protein) was shown by Erdile et al. [3] to induce a protective response and serum immunoglobulin against *B. burgdorferi* infections. Braun and co-workers [4–8] reported first on an outer membrane 7-ku protein from *E. coli* [4]; they described its covalent linkage to peptidoglycan [5, 6], its primary sequence [7, 8], and the covalent binding of a diacylglycerol moiety [9]. Further, Inouye et al. [10] and Hirashima et al. [11] recognized that only one third of this 7 ku lipoprotein is covalently linked via the ε-NH_2 group of the C-terminal lysine to the α-COOH group of the diaminopimelic acid residue of tripeptide side chains of peptidoglycan. The remaining two thirds of this lipoprotein is in the free form. Inouye [12] described a model for three-dimensional molecular assembly of this lipoprotein to form hexameric to octameric protein tubular channels that penetrate the outer membrane.

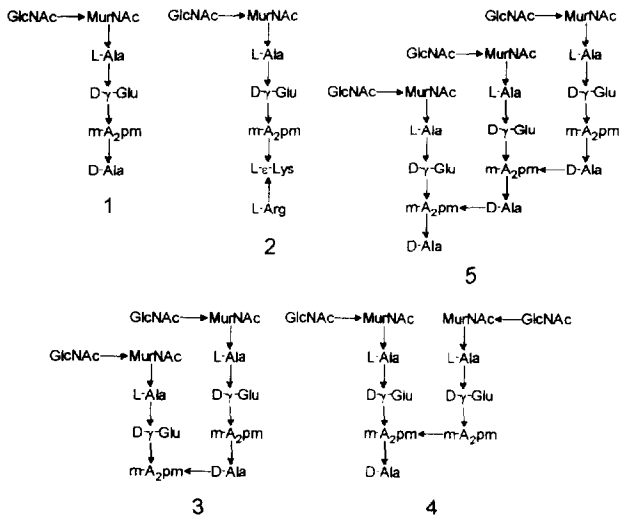


Figure 1. Chemical structures of some major "peptidoglycan" fragments (monomer, dimer, trimer) obtained by consecutive digestion of *E. coli* peptidoglycan with pronase E and *Chalaropsis* muramidase. The structures exhibit unusual alternating L- and D-configured amino acids, unusual linkage for the glutamic acid residues as well as cross linkage of peptide side chains. Examples include the monomers 1 (Tetra) and 2 (Tri–Lys–Arg, one pronase E digestion product of lipoprotein linked to peptidoglycan), two isomeric dimers 3 (Tetra–Tri, D-Ala → D-A_2pm cross linked) and 4 (Tetra–Tri, L-A_2pm → D-A_2pm cross linked), and the trimer 5 (Tetra–Tetra–Tetra).

These two forms of outer membrane 7-ku lipoprotein are both known as Braun's lipoprotein. In addition, a so-called peptidoglycan-associated lipoprotein (PAL) that has a molecular weight of 21 ku was described from *E. coli* present in close association with peptidoglycan but not covalently bound to it [13, 14]. Later, Ichihara et al. [15] reported on seven new lipoproteins localized either in the outer membrane or in the cytoplasmic membrane of *E. coli*; the molecular weights ranged from 16 to 52 ku as determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Among these nine different lipoproteins known from *E. coli*, only the smallest, the 7-ku lipoprotein, occurs in both the free and the peptidoglycan-bound form. During preparation of peptidoglycan from *E. coli* to obtain water-soluble fragments for high-performance liquid chromatography (HPLC) analysis, the bound form of Braun's lipoprotein must be degraded by digestion with pronase E, which leaves the C-terminal end Arg–Lys at tripeptide subunits. These degradation products appear as water-soluble Lys–Arg-containing peptidoglycan fragments [16, 17] in HPLC chromatograms that indicate the various attachment sites of this lipoprotein to peptidoglycan.

We have previously used reverse phase HPLC in "off-line" combination with ^{252}Cf plasma desorption time-of-flight mass spectrometry (^{252}Cf-PD TOF/MS) to study native [18–20] and biosynthetically modified peptidoglycan preparations from *E. coli* [21], the peptidoglycan structure of the cyanelles from *Cyanophora paradoxa* [22, 23], the peptidoglycan from the cyanobacterium *Synecocystis spec.* strain PCC 6714 [24], and the peptidoglycan from the thermophile bacterium *Thermus thermophilus* [24b]. In these investigations we adapted the nitrocellulose adsorption technique, originally described for peptides and proteins [25], to the analysis of strongly hydrophilic, muramidase-released peptidoglycan fragments [26]. A rinsing procedure is absolutely necessary to remove salt contaminations

(especially sodium and potassium cations, which are commonly associated with such hydrophilic glycoconjugates even after extensive chromatographic purification) and to eliminate interference with the relative molecular mass determination of unknown compounds. Rinsing procedures up to several 100 μL of solvent are only tolerated by analytes strongly adsorbed onto nitrocellulose, which is not the case for most peptidoglycan fragments. Therefore we developed a sample preparation technique, the so-called "nitrocellulose sandwich technique" [26], in which the hydrophilic analyte is sandwiched between two layers of nitrocellulose prior to microrinsing; this allows selective removal of salt contaminations even for very hydrophilic compounds without dramatic sample loss.

As an extension of our investigations on different peptidoglycans we sought to establish a method for the mass spectrometric characterization of compounds covalently linked to peptidoglycan, in particular, the lipoproteins of Gram-negative bacteria. The water-insolubility of these proteins poses a number of technical difficulties whose solution also could probably apply to other families of hydrophobic proteins and peptides. As a model system we chose Braun's lipoprotein from *E. coli* because extensive knowledge of this protein facilitates a critical evaluation of the accuracy and reliability of the methods applied. In this paper we describe a strategy that uses two different time-of-flight mass spectrometric techniques for the analysis of the free form of Braun's lipoprotein and its isolated N-terminal lipid anchor. We also describe the determination of peptidoglycan attachment sites of the bound form of this lipoprotein. Results obtained by ^{252}Cf-PD TOF/MS are compared with data obtained with matrix-assisted laser desorption–ionization mass spectrometry (MALDI TOF/MS). Special emphasis in MALDI mass spectrometry was also given to an appropriate sample preparation technique due to the specific properties of these different classes of compounds (lipoproteins, lipids, glycopeptides). The experimental approach presented in this paper also may have a rather wide interest to protein chemists because it should be of direct and general applicability to the analysis of bacterial lipoproteins and peptidoglycan–lipoprotein complexes.

Experimental

Bacterial Strains and Growth Conditions

E. coli MC6 RP1 (K12, F , proA leuA thr dra drm lysA thi) [27] was grown aerobically to the late exponential phase in a medium composed of commercially available tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (5 g/L), magnesium sulfate (1.2 g/L), and glucose (3.6 g/L) in a 200-L fermenter at pH 6.8. Growth of the bacteria was monitored by measuring the optical density at λ = 550 nm. Cells were harvested by centrifugation and were stored frozen at −70 °C until used.

Isolation and Purification of the Free Form of Braun's Lipoprotein

The free form of Braun's lipoprotein was purified by a modification of the procedure described by Inouye et al. [28]. After the second acetone precipitation the lipoprotein pellet was resuspended in 0.01-M ethylenediaminetetraacetic acid (EDTA), 1% SDS in 0.01-M sodium phosphate buffer pH 7.0, and further purified by gel filtration chromatography on a Sephadex G-100 Superfine column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The column (32 × 2.5 cm) was equilibrated and eluted with the same buffer at a flow rate of 12 mL/h. Fractions (1 mL) were collected and the protein content of every fraction was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) that measured the absorbance at λ = 562 nm. Every fraction also was tested for the presence of Braun's lipoprotein by SDS–polyacrylamide gel electrophoresis. All fractions that contained this lipoprotein were pooled. Final removal of salts and SDS was performed by precipitation with acetone. The final pellet was lyophilized and stored at −20 °C.

Cyanogen Bromide Cleavage of the Free Form of Braun's Lipoprotein

Purified lipoprotein (20 mg) was dissolved in 15 mL of formic acid (Merck, Darmstadt, Germany) that contained 20% ultrapure water obtained from a MilliQ plus aperture (Millipore, Millford, MA; conductivity, 18.2 MΩ/cm) to which finally 600 mg of colorless cyanogen bromide (Merck) was added. The cleavage was performed under a nitrogen atmosphere in the dark at room temperature for 24 h at 20 °C. The cleavage reaction was terminated by evaporation to dryness via a Univapo vacuum centrifuge (Univapo, Martinsried, Germany). Separation of the water-insoluble fraction, which contained mainly the N-terminal lipopeptide, from the water-soluble fraction was performed by the addition of 5 mL of ultrapure water to the dried cleavage products and centrifugation at 10.000 g for 10 min. Both fractions were separately dried by lyophilization.

HPLC Separation of the Water-Soluble Peptides Released by Cyanogen Bromide Cleavage of the Free Form of Braun's Lipoprotein

Approximately 100 μg of the water-soluble peptides of the cyanogen bromide digest were separated by HPLC by using a Spectra-Physics SP8100XR extended range liquid chromatographer (Spectra Physics Analytical, Fremont, CA) connected to a Spectra Physics SP 4270 integrator and a Millipore Waters Lambda-Max Model

481 liquid chromatography (LC) spectrophotometer (Millipore Waters, Millford, MA). The stationary phase was Hypersil ODS (Shandon, Runcorn, UK) with 3-μm particle size packed in a 250 × 4-mm column (Teknochroma, Barcelona, Spain). The mobile phase consisted of 0.1% trifluoroacetic acid in ultrapure water as solvent A and acetonitrile (Scharlau, Barcelona, Spain) and ultrapure water (7:3) that contained 0.1% trifluoroacetic acid as solvent B. The column temperature was maintained at 25 °C and the flow rate was 0.5 mL/min. The detection wavelength λ was 214 nm. The gradient was linear from 0 to 50% solvent B (0–50 min) and then from 50 to 100% solvent B (50–75 min). Manually collected fractions of interest were immediately evaporated to dryness and kept at −20 °C until further use.

Isolation and Purification of the Bound Form of Braun's Lipoprotein and Subsequent Purification of Lys–Arg-Containing Peptidoglycan Fragments

Peptidoglycan from *E. coli* MC6 RP1 was purified following standard procedures [16, 17] by consecutive digestion with α-amylase type II-A from *Bacillus spec.* (Sigma, St. Louis, MO) and muramidase from *Chalaropsis spec.* as reported [29] except that pronase E was used previously. By treatment of the intact peptidoglycan with muramidase, all peptidoglycan fragments not attached to lipoprotein are solubilized in water (fraction **1**). This fraction therefore is separated easily from the water-insoluble fraction that includes lipoprotein-attached peptidoglycan fragments as well as the remaining undigested peptidoglycan. To remove the undigested peptidoglycan, the pellet was boiled in 5% SDS solution to dissolve the bound form of the lipoprotein (fraction **2**). After centrifugation at 10.000 g for 20 min sufficient acetone was added to the supernatant to precipitate purified lipoprotein again. The lipoprotein pellet was resuspended three times in water to remove remaining SDS and finally lyophilized. To obtain almost all of the Lys–Arg-containing peptidoglycan fragments that occur in sufficient amount, fraction **2** was subsequently digested with preactivated pronase E from *Streptomyces griseus* (Merck) by suspension of the analyte in 10-mM Tris (Merck) buffer pH 7.2. To the resulting mixture of soluble Lys–Arg-containing peptidoglycan fragments, a sodium borate buffer pH 8 was added and reduction was carried out using sodium borohydride (Merck) for 30 min. The reaction was stopped by adjusting the pH of the reaction mixture to 4 by the addition of concentrated phosphoric acid (Merck).

Finally, the peptidoglycan fragments were purified from pronase E degradation products by using the same HPLC equipment as described previously with the following mobile phases: solvent A, 50-mM sodium phosphate pH 4.35; solvent B, 75-mM sodium phosphate pH 4.95 that contained 15% methanol. The solvent used was 100% solvent A for 7 min, then a gradient from 0 to 100% solvent B (7–122 min), and finally 100% solvent B (122–150 min). The flow rate was 0.5 mL/min and the column temperature was 40 °C. The detection wavelength was 210 nm. Preliminary identification of Lys–Arg-containing peptidoglycan fragments was based on comparing HPLC profiles of the native peptidoglycan fragment mixture, fraction **1** and fraction **2** (all sodium borohydride-reduced). Only peaks that appear in the native mixture and in fraction **2**, but not in fraction **1**, were selected as possible candidates for Lys–Arg-containing peptidoglycan fragments.

Isolation of the Posttranslationally Modified Cysteine of Braun's Lipoprotein

The intact purified free form of Braun's lipoprotein was resuspended in 10-mM Tris (Merck) buffer pH 7.2 and digested with preactivated pronase E for 12 h (enzyme to substrate ratio, 1:1). Final extraction of the extremely hydrophobic lipid anchor (= *S*-[2,3-bis(acyloxy)-propyl-]-*N*-acylcysteine) was performed by direct extraction of the enzymatic digest solution with chloroform (Merck). For the plasma desorption (PD) mass spectrometric analysis the extracted lipid anchor was used without further purification.

^{252}Cf Plasma Desorption Time-of-Flight Mass Spectrometry

All analyses were performed on a BioIon 20 plasma desorption time-of-flight mass spectrometer (BioIon, Uppsala, Sweden) fitted with a standard linear flight tube (150 mm) and 10-μCi ^{252}Cf ion source. Due to sufficient adsorption properties of most compounds investigated, nitrocellulose (BioRad, Richmond, CA) was used as support material [25]. Approximately 100 μg of nitrocellulose dissolved in acetone (1 μg/μL) was electrosprayed onto aluminized Mylar targets. All positive-ion PD mass spectra were obtained at an accelerating voltage of +20 kV and a time resolution of 1 ns per channel. Intact lipoprotein and the cyanogen bromide-generated N-terminal lipopeptide samples were adsorbed from a solution that contained formic acid:water:methanol:2-propanol = 5:15:40:40, similar to the solvent system described by Bouchon et al. [30] for electrospray ionization mass spectrometry (ESI/MS). Water-soluble cyanogen bromide-generated peptide fragments and peptidoglycan fragments were adsorbed from aqueous 0.1% trifluoroacetic acid solutions. Spectrum acquisition times were 2×10^7 primary events for the intact lipoprotein samples and 5×10^6 primary events for the cyanogen bromide-generated peptides as well as for all types of peptidoglycan fragments. Prior to analysis all samples where rinsed with 3 × 100 μL (in the case of the intact lipoprotein and peptide fragments) or 3 × 10 μL (in

the case of peptidoglycan fragments) of 0.1% trifluoroacetic acid in ultrapure water to remove interfering alkali cations and other low mass contaminants. The free form of Braun's lipoprotein and the cyanogen bromide-derived N-terminal lipopeptide were also investigated by using 9-anthroic acid (Fluka, Buchs, Switzerland) and 3-(3-indolyl) acrylic acid (Sigma) matrix (Pittenauer, E. et al., manuscript in preparation). The advantage of this matrix compared to nitrocellulose support has been reported [31] to be a lesser internal energy of desorbed/ionized molecules that results in decreased metastable decompositions during the flight and therefore in improved peak shapes of protonated ions. Interestingly, this matrix recently was described by Chou et al. [32] as a useful matrix for MALDI mass spectrometry of dynorphin peptides. Approximately 210–300 μL of a solution of 9-anthroic acid (ACA) or 3-(3-indolyl) acrylic acid (IAA) in acetone (each 1 μg/μL) was electrosprayed onto aluminized Mylar backings. Prior to sample adsorption the matrix surface was rinsed with 3 × 100-μL 0.1% trifluoroacetic acid in ultrapure water. Sample adsorption was performed from the same solvent mixture as for the nitrocellulose support. Prior to analysis all samples were rinsed with 3 × 100-μL 0.1% trifluoroacetic acid in ultrapure water.

For the PD mass spectrometric characterization of the pronase E-released lipid anchor (which is soluble only in chloroform) nitrocellulose support, IAA and ACA as the matrix could not be used due to disintegration of the support/matrix layers when this solvent was applied. The matrix material 3-(3-pyridyl) acrylic acid (PAA; Aldrich, Steinheim, Germany) recently was compared with nitrocellulose support and found to yield superior results for several different classes of lipids and glycolipids in positive- as well as negative-ion mass spectra [20, 33–35]. PAA shows high surface stability against chloroform as an analyte solvent. Approximately 300 μL of a saturated solution of PAA in acetone was electrosprayed onto an aluminized Mylar target. Prior to sample deposition the matrix surface was rinsed with 3 × 100-μL ultrapure water. Finally, a 10-μL aliquot of the free lipid anchor (between 1 and 10 μg of lipid) dissolved in chloroform was deposited onto the matrix surface. To remove alkali cations and other low mass contaminations selectively, the sample was rinsed with 3 × 50-μL ultrapure water prior to analysis.

Matrix-Assisted Laser Desorption–Ionization Time-of-Flight Mass Spectrometry

Mass spectrometric measurements were performed in the reflector mode on a Kratos Analytical Kompact MALDI-III (Kratos Analytical, Manchester, UK) laser desorption time-of-flight instrument equipped with a nitrogen UV laser (λ = 337 nm, 3-ns pulse width). Positive-ion mass spectra were obtained by signal averaging of 100 laser shots. During data acquisition the shot-to-shot signal was viewed on the computer monitor as the laser was attenuated to maintain the optimal laser power density near threshold level ($\sim 10^6$ W/cm^2). The ions were accelerated to a final potential of +20 kV. Conversion of flight time to mass-to-charge ratio was achieved by internal calibration by using the $[M - OH]^+$ peak of the matrix (m/z 207.21) and the $[M + H]^+$ peak of bovine insulin. Mass assignments for the intact free form of Braun's lipoprotein were derived from the centroided top 30% of the ion signals. Sample preparation was done according to the recently reported procedure for bacteriopsin [36] with sinapinic acid matrix (Sigma). Additionally, Lys–Arg-containing peptidoglycan fragments were measured using 2,5-dihydroxybenzoic acid matrix as recently described for

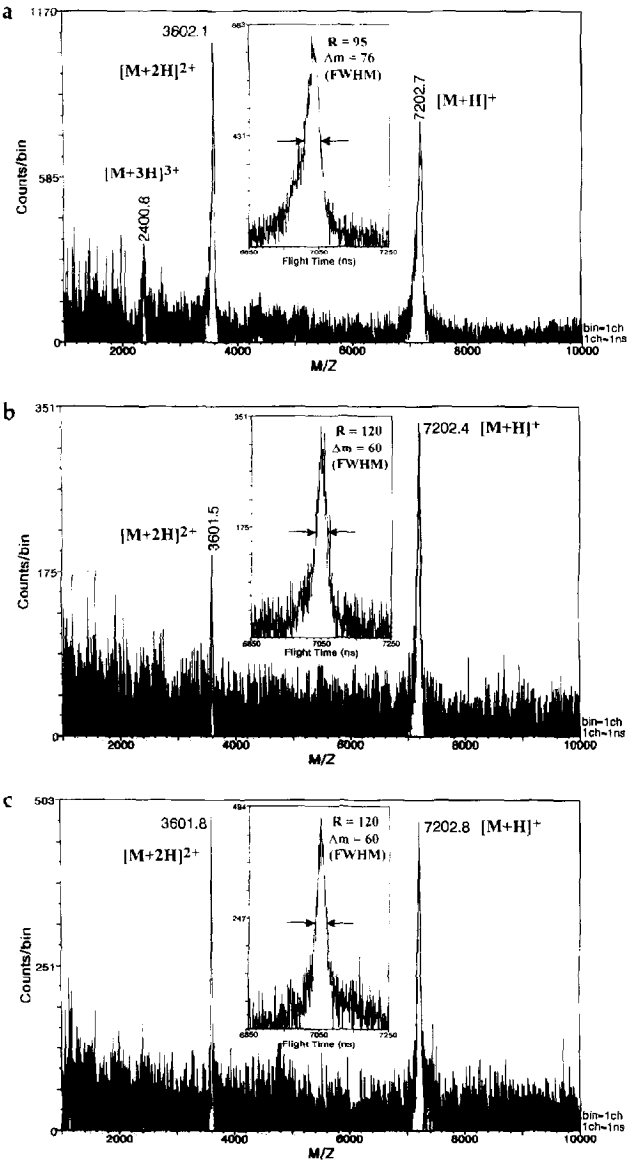


Figure 2. Positive-ion PD mass spectra of the free form of Braun's lipoprotein from *E. coli* MC6RP1 adsorbed onto (**a**) nitrocellulose support, (**b**) 9-anthroic acid matrix, and (**c**) 3-(3-indolyl) acrylic acid matrix.

Table 1. Molecular weight determination of the intact free Braun's lipoprotein[a]

Ionization technique	Support or matrix	$[M + H]^+/[M + 2H]^{2+}/[M + 3H]^{3+}$ average m/z values		Difference between observed and calculated molecular weight expressed as Δm based on n types of ions	Mass deviation (%)
		Calculated	Found		
PD	NC	7202.70/	7202.7/	−0.61 ($n = 3$)	−0.0085
		3601.85/	3602.1/		
		2401.57	2400.8		
PD	ACA	7202.70/	7202.6/	+1.50 ($n = 2$)	+0.0208
		3601.85/	3603.4/		
		2401.57	(—)		
PD	IAA	7202.70/	7202.8/	0 ($n = 2$)	—
		3601.85/	3601.8/		
		2401.57	(–)		
MALDI	SA	7202.70/	7203.8/	−0.40 ($n = 2$)	+0.0056
		3601.85/	3601.2/		
		2401.57	(—)		

[a]Primary sequence as reported by Braun and Bosch [7, 8] C*SSNAKIDQLSSDVQTLNAKVDQLSNDVNAMRSDVQAAKDDAARANQRLDN-MATKYRK; C* corresponds to the lipid-modified cysteine moiety. Average calculated molecular weight is based on the major lipid composition 18:1 -16:0-16:0; 7201.69.) by positive-ion PD TOF/MS and MALDI TOF/MS. Abbreviations used: NC, nitrocellulose; ACA, 9-anthroic acid; IAA, 3-(3-indolyl) acrylic acid; SA, sinapinic acid.

peptides and proteins [37]. The sample/matrix mixtures were applied in the center of the disposable sample probe and the solvents were removed slowly in the system apparatus before insertion into the ion source. The lipid anchor did not yield any positive or negative quasimolecular ion when sinapinic acid or 2,5-dihydroxybenzoic acid were used. Application of the lipid anchor directly onto the probe tip without any matrix would most likely result in detection of a negative-ion cluster that corresponds to $[M - COOH]^-$ ions.

Results and Discussion

Until recently molecular weight determination of strongly hydrophobic, water-insoluble membrane proteins was a difficult task for desorption–ionization mass spectrometric techniques due to the insolubility of these proteins in many solvents and buffers compatible with mass spectrometry. Up to now only a few examples of molecular weight determination of such hydrophobic proteins as well as their cyanogen bromide-generated peptide fragments have been reported (e.g., bacteriopsin and bacteriorhodopsin from *Halobacterium halobium* and other sources by MALDI TOF/MS [36] and ESI/MS [38–40]; cyanogen bromide peptide fragments of native bacteriopsin by ESI/MS [41] as well as biosynthetically modified bacteriopsins by fast-atom bombardment mass spectrometry (FAB/MS) [42]; a genetically engineered 28-ku lipoprotein from *B. burgdorferi* [30] and a thermolytic fragment of colicin A by ESI/MS [43]; cyanogen bromide peptide fragments from chicken intestinal calbindin-D^{28k} by ^{252}Cf-PD TOF/MS [44]).

Most applications of protein molecular weight determination by ^{252}Cf-PD TOF/MS within the last decade were performed via analyte adsorption onto nitrocellulose support from acidified aqueous solutions. So far no experiments have been reported on membrane protein adsorption from analyte solutions that contain a high percentage of organic solvents (> 80%) such as adsorption from alcoholic solvents onto support or matrix materials. (The distinction between support and matrix materials has been made by Roepstorff [45].) In an attempt to solubilize Braun's lipoprotein, we adopted a solvent system similar to that described by Bouchon et al. [30] for the ESI/MS analysis of the 28-ku outer surface protein A (OspA) from *B. burgdorferi*. Roughly 1 nmol of lipoprotein was adsorbed from this high percentage alcoholic solution onto nitrocellulose support or onto 9-anthroic acid- or 3-(3-indolyl) acrylic acid matrix for several minutes without visible disintegration of the support/matrix surface. After an extensive rinsing procedure to re-

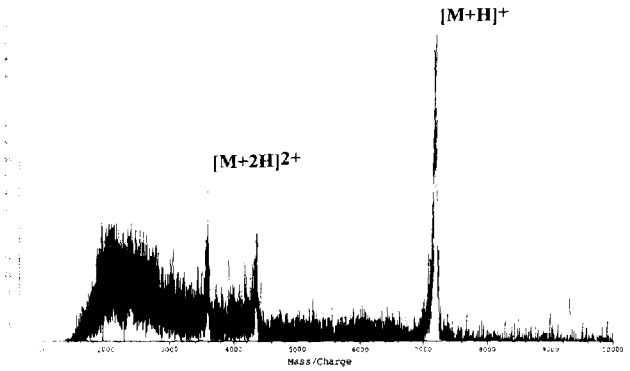
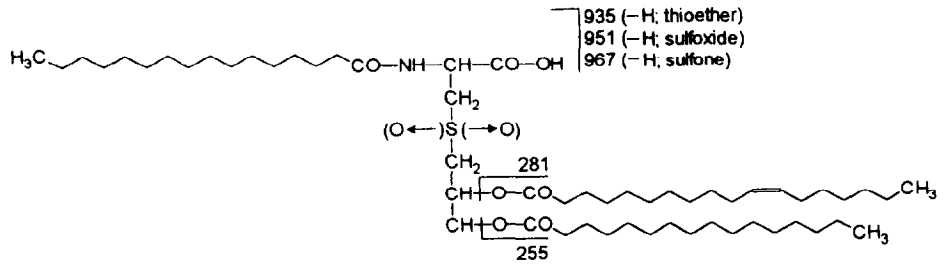


Figure 3. Positive-ion MALDI mass spectrum of the free form of Braun's lipoprotein from *E. coli* MC6RP1 utilizing sinapinic acid matrix. The small peak at m/z 4200 most likely corresponds to an unknown impurity.

Table 2. Chemical structure of the major component of the isolated lipid anchor (*S*-[2,3-bis(acyloxy)-propyl-]-*N*-acylcysteine) released by pronase E digestion of the free form of Braun's lipoprotein and molecular species detected in the negative- and positive-ion PD mass spectrum[a]



Fatty acid composition of the *S*-[2,3-bis(acyloxy) -propyl-]-*N*-acylcysteine moiety	Average molecular weight	[M − H]⁻		[M + PAA + H]⁺	
		Calculated	Found	Calculated	Found
16:0-16:0-16:1	**908.46**	**907.45**	**907.2**	**1058.62**	**1058.8**
16:0-16:0-16:1 sulfoxide	924.46	923.45	923.0	1074.61	1073.5
18:1-16:0-16:0	**936.51**	**935.50**	**935.0**	**1086.67**	**1086.4**
18:1-16:0-16:0 sulfoxide	952.51	951.50	951.0	1102.67	1101.3
18:1-18:1-16:0	**962.55**	**961.54**	**961.0**	**1112.71**	**1114.4**
18:1-18:1-16:0 sulfoxide	978.55	977.54	977.2	1128.71	1128.3
18:1-18:1-16:0 sulfone	994.55	993.54	992.6	1144.71	—

[a]Only boldface type corresponds to native molecules. Sulfoxides and sulfones are most probably artifacts due to oxidation during preparation of the lipid anchor.

move interfering alkali contaminants, positive-ion PD mass spectra of good quality were obtained. The results confirmed the calculated molecular weight based on the published primary sequence [7, 8] and the expected [9] 18:1-18:1-16:0 fatty acid composition for the lipid anchor as the major molecular species (see Figure 2a–c; see also Table 1 for mass accuracies obtained with different sample preparation techniques). Interestingly, no triply protonated molecular ions for this lipoprotein were observed in the PD mass spectrum when the small molecule matrix materials 9-anthroic acid or 3-(3-indolyl) acrylic acid were used.

Analysis of < 5 pmol of the intact free form of Braun's lipoprotein by MALDI TOF/MS with sinapinic acid as the matrix gave a comparable result with similar mass accuracy to PD TOF/MS (see Figure 3 and Table 1).

Three factors prevented the detection of minor molecular species that vary in only one fatty acid substituent (Δm = 26 for a —CH=CH— or 28 u for a $—CH_2—CH_2—$ moiety for the fatty acid alkyl side chain): (1) the relatively high molecular weight of the lipoprotein sample; (2) a certain degree of peak broadening by metastable decay of the protonated molecular ions; (3) insufficient mass resolution performance of the available linear PD time-of-flight instrument as well as the reflector MALDI time-of-flight instrument in the 7-ku mass range. To investigate the heterogeneity of the fatty acid moieties of the lipid anchor, the

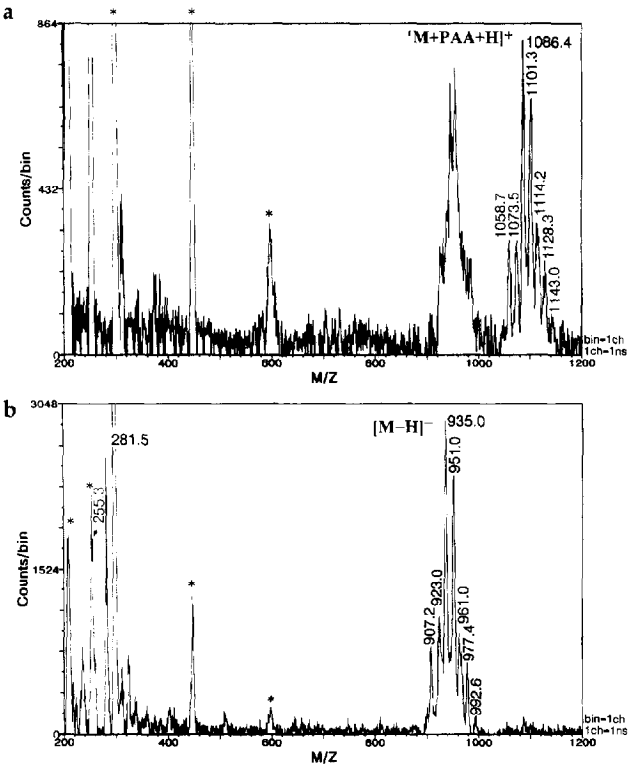


Figure 4. (**a**) Positive- and (**b**) negative-ion PD mass spectra of the pronase E-released isolated lipid anchor (*S*-[2,3-bis(acyloxy)-propyl-]-*N*-acylcysteine) deposited onto 3-(3-pyridyl) acrylic acid matrix. Asterisks (*) correspond to PAA matrix cluster ions.

intact free form of Braun's lipoprotein was digested with pronase E to achieve nonspecific hydrolysis of all L,L-configured peptide bonds of the protein and to leave mainly free amino acids. Only the N-terminal amino acid cysteine covalently linked to the lipid moiety exhibited superior solubility in chloroform to allow direct and selective extraction of the lipid anchor into the organic solvent phase. The crude chloroform extract was analyzed without further purification by positive- and negative-ion PD TOF/MS. 3-(3-Pyridyl) acrylic acid was selected as the matrix for this diglyceride thioether lipid because of strong chemical similarities to glycerol ester-based lipids and phospholipids that have been successfully analyzed by utilizing this matrix [33–35]. In the positive-ion PD mass spectrum no protonated molecular ion peaks could be detected due to the absence of any strong protonation site in the molecule (Figure 4a). Instead, abundant adduct ions with PAA matrix of the type $[M + PAA + H]^+$ that showed no significant fragmentation were detected. The very broad peak cluster centered around m/z 950 corresponds most likely to metastable ions of the $[M + PAA + H]^+$ adduct ions and represents ions with m/z values not directly correlated with any protonated molecular ion or fragment ion. In the negative-ion PD mass spectrum of the lipid anchor (Figure 4b) very abundant deprotonated molecular ions were formed: m/z 907.2, 16:0–16:0–16:1; m/z 935.0, 18:1–18:1–16:0, and m/z 961.0, 18:1–18:1–18:1 are observed as major components. Furthermore, abundant fatty acid fragment anions at m/z 281.5 (18:1, *cis*-vaccenate) and m/z 255.3 (16:0, palmitate) correlated with the major fatty acid constituents of the lipid anchor were detected [9].

Additionally, as the result of digestion of Braun's lipoprotein with pronase E at roughly neutral pH conditions, partial oxidation of this native mixture of homologous compounds takes place at the thioether moiety to the corresponding sulfoxide and sulfone derivatives, respectively, of the *S*-[2,3-bis(acyloxy)-propyl]-*N*-acylcysteines (see Table 2). No mass shift by +16 and +32 u, respectively, of the protonated molecular ion of the intact free form of this lipoprotein was observed in the positive-ion PD mass spectrum (compare Figures 2 and 3).

MALDI mass spectrometry failed to give any useful results in either ionization mode with either 2,5-dihydroxybenzoic acid or sinapinic acid. Only in the absence of any UV-absorbing matrix material could a negative-ion MALDI mass spectrum be obtained where detected negative ions corresponded most likely to decarboxylated lipid anchor anions (data not shown)

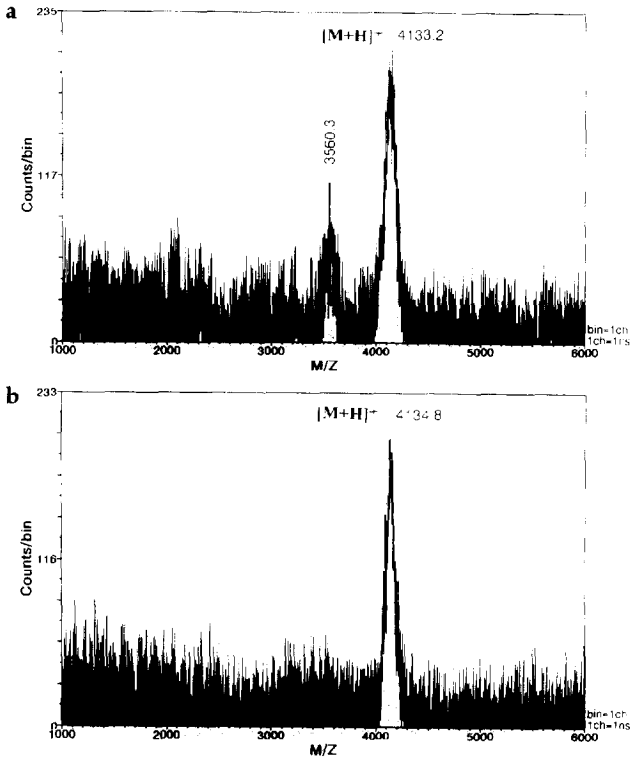


Figure 5. Positive-ion PD mass spectra of the water-insoluble cyanogen bromide-released N-terminal lipopeptide CNBr 1 from the outer membrane 7-ku lipoprotein adsorbed onto (**a**) nitrocellulose support and (**b**) 9-anthroic acid matrix.

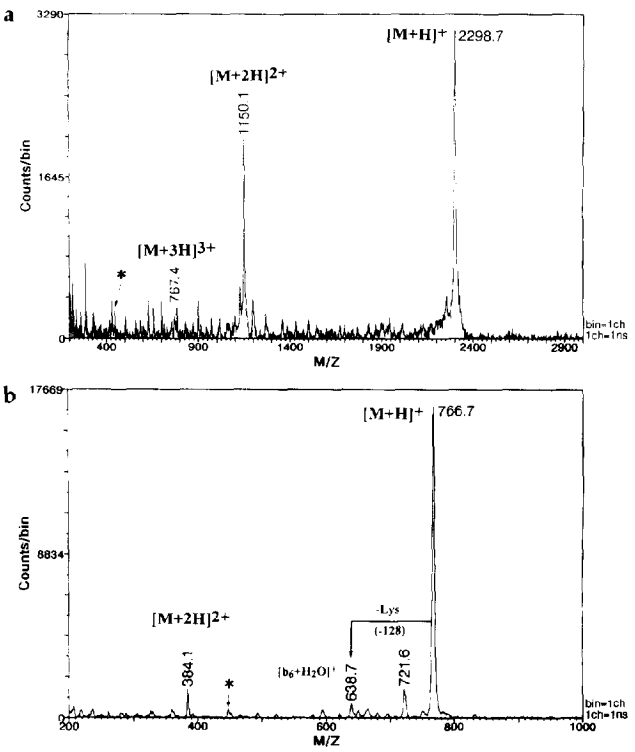


Figure 6. Positive-ion PD mass spectra of the water-soluble and reverse phase HPLC-separated cyanogen bromide-released peptides from the free form of Braun's lipoprotein representing (**a**) CNBr 2 and (**b**) CNBr 3, both adsorbed onto nitrocellulose support. An asterisk (*) corresponds to the $M^{+\cdot}$ radical molecular ion of a polyphenyl ether component from the diffusion pump oil; elemental composition $C_{30}H_{22}O_4$; average calculated molecular weight 446.50.

that represent a similar pattern to that observed in PD TOF/MS.

To check the complete primary sequence of the free form of Braun's lipoprotein the protein was cleaved with cyanogen bromide in formic acid as solvent (experiments that involved trifluoroacetic acid as solvent to prevent possible formylation of the resulting peptides yielded unsatisfactory results compared to formic acid). Due to the presence of two methionines in the amino acid sequence three cleavage products were expected, which include two water-soluble peptides as well as one water-insoluble peptide that corresponds to the N-terminal lipopeptide. Based especially on the solubility properties of the lipopeptide, the whole mixture was dissolved in the same solvent as that used for the intact lipoprotein for sample adsorption onto nitrocellulose support. The resulting positive-ion PD mass spectrum exhibited all peptide fragments, which included the strongly hydrophobic lipopeptide (CNBr 1), but with very low abundance and some singly and doubly formylated side reaction products (data not shown). Therefore the whole peptide mixture was separated first into a water-insoluble fraction (that contained mainly the lipopeptide, CNBr 1) and a water-soluble fraction (that contained the other two peptides, CNBr 2 and CNBr 3) by a simple centrifugation procedure by using aqueous 0.1% trifluoroacetic acid as solvent. The pellet (the water-insoluble fraction) mainly contained the doubly formylated lipopeptide, as confirmed by positive-ion PD TOF/MS (see Figure 5). The soluble part that contained the remaining two cyanogen bromide-generated peptides was further purified and separated by reverse phase HPLC. Mainly nonformylated peptides, but also traces of singly formylated products, were observed as individual LC peaks identified by positive-ion PD TOF/MS (Figure 6 and Table 3). Comparison of the calculated values for the cyanogen bromide peptides with the found values showed that only the $[M + H]^+$ ion of the lipopeptide exhibited a significant mass shift of +56 u (Table 3). This shift corresponds most likely to a doubly formylated lipopeptide $[M + H]^+$ ion, a common artefact of peptides and proteins cleaved with cyanogen bromide in the presence of a high percentage of formic acid as solvent [38, 42]. Formylation of peptides normally occurs first at the N-terminal end, but due to the fact that this lipopeptide is N-terminally blocked with a fatty acid moiety, formylation can take place only at the amino acid side chains of this peptide (e.g., serine, threonine, and lysine). The fragment ion m/z 3560.3 in Figure 5a, where the lipopeptide is adsorbed onto a nitrocellulose support, most likely corresponds to loss of the diglyceride moiety, which is not detected when the sample is adsorbed onto 9-anthroic acid matrix (Figure 5b) due to lower internal energy of desorbed–ionized sample molecules [31].

Abundant fragment ions correlated each with their primary sequence were detected during analyses of the internal lipoprotein sequence peptide CNBr 2 (Figure 6a) and the C-terminal hexapeptide CNBr 3 (Figure

Table 3. Molecular weight determination of the peptide fragments generated by cleavage of the free form of the outer membrane 7-ku lipoprotein with cyanogen bromide as well as the by-products (e.g., formylation, hydrolysis of the C-terminal homoserine lactone) known to be present in such digests; the fragments were separated by reverse phase HPLC

HPLC fraction number R_t (min)	CNBr fragment	$[M + H]^+/[M + 2H]^{2+}/[M + 3H]^{3+}$ average m/z values		Amino acid sequence	Primary sequence of the CNBr peptide fragment[a]
		Calculated	Found		
Water-insoluble	CNBr 1	4077.18/	(—)/	1–31	C*SSNAKIDQLSSDVQTLNAKV
		2039.09/	(—)/		DQLSNDVNAHsl
		1360.07	(—)		
Water-insoluble	CNBr 1, diformylated	4133.20/	4133.2/	1–31	C*SSNAKIDQLSSDVQTLNAKV
		2067.10/	(—)/		DQLSNDVNAHsl
		1378.74	(—)		(diformylated)
1 (35.04)	CNBr 3	766.92/	766.8/	53–58	ATKYRK
		383.96	383.8		
2 (38.50)	CNBr-3, monoformylated	794.93/	794.8/	53–58	ATKYRK (monoformylated)
		397.97	397.8		
3 (47.61)	CNBr-2	2299.42/	2298.7/	32–52	RSDVQAAKDDAARANQRLDN
		1150.22/	1150.1/		Hsl
		767.15	767.4		
4 (49.42)	CNBr 2, monoformylated	2327.43/	2325.7/	32–52	RSDVQAAKDDAARANQRLDN
		1164.22	1163.9		Hsl (monoformylated)
5 (49.42)	CNBr 2, monoformylated and hydrolyzed	2345.45/	2344.7/	32.52	RSDVQAAKDDAARANQRLDN
		1173.23	1172.9/		Hse (monoformylated and hydrolyzed)

[a] C* = *S*-[2,3-bis(acyloxy)-propyl]-*N*-acylcysteine. m/z values calculated for C*-containing peptides are based on a 16:0–16:0–18:1-major fatty acid composition of the lipid anchor. Hsl corresponds to C-terminal homoserine lactone. Hse corresponds to C-terminal homoserine.

Table 4. Fragment ions of the cyanogen bromide-generated peptide CNBr 2 observed in the positive-ion PD mass spectrum (compare Figure 6b); $[M + H]^+ = 766.92$ (calc.)[a]

Peptide fragment ion type	m/z Value		Mass deviation (Δm)
	Calculated	Found	
$z_2/z_2 + 1$	286.35/287.36	287.35	+1.00/− 0.0
y_2	303.38	303.45	+0.07
x_2	329.38	329.33	−0.05
w_3	358.41	358.61	−0.20
$z_3/z_3 + 1$	449.53/450.54	450.33	+0.80/− 0.21
y_3	466.56	466.10	−0.46
x_3	492.56	492.35	−0.21
v_4	520.61	521.61*	+1.00
w_4	521.60	521.61*	+0.01
$z_4/z_4 + 1$	577.70/578.71	578.89	+1.19/+ 0.18
$a_5/a_5 + 1$	592.71/593.73	593.67	+0.96/− 0.06
y_4	594.73	593.67	−1.06
x_4	620.73	620.41*	−0.32
b_5	620.73	620.41*	−0.32
$b_5 - H_2O$	638.74	638.67	−0.07
w_5	649.77	649.75	−0.02
v_{5a}/v_{5b}	662.81/664.78	664.18*	+1.37/− 0.60
d_6	663.80	664.18*	+0.38
$z_5/z_5 + 1$	678.81/679.82	679.16	+0.35/− 0.66
—K	694.79	695.15*	+0.36
y_5	695.84	695.15*	−0.69
$a_6/a_6 + H$	720.89/721.90	721.16*	+0.27/− 0.74
x_5	721.83	721.16*	−0.67
b_6	748.90	750.14*	+1.24
$z_6/z_6 + 1$	749.89/750.90	750.14*	+0.25/− 0.76
w_6	750.88	750.14*	−0.74

[a]An asterisk (*) indicates possible peak overlapping of fragment ions of similar mass value due to insufficient mass resolution of this time-of-flight instrument.

6b). The fragment ions included both peptide backbone as well as peptide side chain fragment ions classified according to the modified Roepstorff–Fohlman nomenclature [46, 47] as earlier described by several groups for positive-ion PD TOF/MS [48–63] (Tables 4 and 5).

Additionally, the C-terminal hexapeptide exhibited a peptide backbone rearrangement ion of type $[b_5 + H_2O]^+$ by loss of the C-terminal amino acid lysine (see Figure 6b). This rearrangement ion represents a fragmentation type previously reported from high energy collision-induced dissociation (CID) spectra of alkali cationized peptides (e.g., $[M + Li]^+$ and $[M + Na]^+$) in three- and four-sector tandem instruments [64–67], as well as from low energy CID spectra of protonated peptides in sector-quadrupole hybrid tandem instruments [68]. It has not, to our knowledge, been reported previously from positive-ion PD mass spectra of peptides. This type of fragment ion of either alkali-cationized or protonated peptides is especially helpful in identifying and confirming the C-terminal amino acid of a peptide. The peptide sequence requirements (at least a free COOH moiety at the C-terminal end) to observe such peptide rearrangement ions in CID spectra already have been discussed in the previously cited papers [64–68].

Finally, an attempt was made to characterize the bound form of Braun's lipoprotein, which means the free form of the Braun's lipoprotein covalently linked to peptidoglycan monomers, dimers, and trimers as well as their corresponding mono-anhydro muramic acid derivatives, that are released after muramidase digestion of native peptidoglycan and differ in mass from the free form by roughly +1000 u to appear in the mass range between m/z 8300 and 10,300. PD TOF/MS and MALDI TOF/MS analyses were tried with the same sample solvent and support/matrix materials used for the free form. Unfortunately, for unknown reasons no useful mass spectra could be obtained from this mixture of "lipoglycoproteins" by either desorption–ionization technique. Therefore, by digesting the bound form of Braun's lipoprotein with pronase E, the complete protein part was hydrolyzed and resulted in a rather complex mixture of Lys–Arg-containing peptidoglycan monomers, dimers, and trimers as well as their corresponding mono-anhydro

derivatives. These components reflected all possible attachment sites of this lipoprotein to peptidoglycan. Separation of this mixture by reverse phase HPLC with subsequent identification of the fractions by positive- and negative-ion PD TOF/MS showed at least seven different Lys–Arg-containing peptidoglycan fragments. Isomeric mono-anhydro dimers and trimers were not separated sufficiently by this reverse phase HPLC system; therefore only isomeric mixtures were measured by PD TOF/MS (Table 6).

Figure 7 shows an example of the positive-ion PD mass spectra of muramidase-generated Lys–Arg-containing peptidoglycan trimer as well as its corresponding isomeric mixture of mono-anhydro trimers; the former and the latter differ in mass by 20 u. The spectra were obtained by using nitrocellulose support after application of a rinsing procedure with water to remove alkali cation contaminations. In contrast to Lys–Arg-containing dimers as well as trimers, which do not exhibit significant fragmentation in PD TOF/MS, the corresponding Lys–Arg-containing peptidoglycan monomer exhibits extensive fragmentation as discussed elsewhere [20]. Figure 8 shows an example of a Lys–Arg-containing peptidoglycan dimer Tri–Tri–Lys–Arg analyzed by positive-ion MALDI TOF/MS without any rinsing procedure. The absence of any sodium or potassium adduct ion formation demonstrates the tolerance of this technique to alkali contaminants. The small peak $[M_2 + H]^+$ at m/z 2080, observed in addition to the $[M_1 + H]^+$ peak of Tri–Tri–Lys–Arg (m/z 2009), corresponds to traces of Tetra–Tri–Lys–Arg that elute next to the former compound and are separated by +71 u. Interestingly, Lys–Arg-free peptidoglycan fragments (that is, peptidoglycan fragments not covalently linked to lipoprotein) yield abundant alkali metal adduct ions in their positive-ion MALDI mass spectra; protonated molecules are absent or of low abundance due to the absence of any strong protonation site in these molecules [18].

Table 5. Fragment ions of the cyanogen bromide released peptide CNBr 3 observed in the positive-ion PD mass spectrum (compare Figure 6a), $[M + H]^+ = 2299.42$ (calc.)[a]

Peptide fragment ion type	m/z Value		Mass deviation (Δm)	Peptide fragment ion type	m/z Value		Mass deviation (Δm)
	Calculated	Found			Calculated	Found	
d_2	200.26	200.46	+0.20	d_{13}	1272.36	1272.72	+0.36
a_2/a_2+1	216.26/217.27	216.15	−0.11/−1.12	$a_{13}/a_{13}+1$	1357.47/1358.48	1357.56	+0.09/−0.92
c_2	261.30	261.09	−0.21	b_{13}	1385.48	1382.48	−3.00
d_3	287.34	287.53	+0.19	$z_{13}/z_{13}+1$	1426.44/1427.45	1427.44	+1.00/−0.01
a_3/a_3+1	331.35/332.36	331.52	+0.17/−0.84	x_{14}	1469.47	1469.08	−0.39
d_4	416.46	416.79	+0.35	w_{14}	1498.51	1498.20*	−0.31
a_4/a_4+1	430.48/431.49	430.74	+0.26/−0.75	d_{15}	1499.63	1498.20*	−0.43
d_5	501.56	501.86	+0.30	$a_{15}/a_{15}-1$	1542.65/1543.66	1543.39	+0.74/−0.27
a_5/a_5+1	558.61/559.62	558.78	+0.17/−0.84	y_{14}	1570.66	1569.69*	−0.97
b_5	586.62	586.38	−0.24	b_{15}	1571.65	1569.69*	−1.96
a_6/a_6+1	629.69/630.70	629.63	−0.06/−1.07	$z_{14}/z_{14}-1$	1597.64/1598.65	1597.30	−0.34/−1.35
b_6	657.70	657.45	−0.25	$z_{15}/z_{15}+1$	1625.70/1626.71	1623.99	−1.71/2.72
a_7/a_7+1	700.77/701.78	700.71	−0.06/−1.07	$a_{16}/a_{16}-1$	1671.77/1672.78	1671.79	+0.02/+0.99
b_7	728.78	728.68	−0.10	x_{15}	1696.77	1697.20	+0.57
d_8	771.85	771.90	−0.05	x_{16}	1739.80	1739.01	−0.79
a_8/a_8+1	828.95/829.96	829.04	−0.09/−0.91	v_{17}	1767.85	1766.15	−1.70
d_9	900.03	900.13	+0.10	$a_{17}/a_{17}-1$	1827.95/1828.96	1827.18	−0.77/−1.78
y_8	914.95	913.95	−1.00	x_{17}	1867.93	1865.85	−2.08
a_9/a_9+1	944.04/945.05	944.04	0/−1.01	d_{18}	1901.05	1898.43	−2.62
b_9	972.05	971.88	+0.83	y_{18}	1941.07	1940.18*	−0.99
d_{10}	1015.11	1015.02	−0.09	$a_{18}/a_{18}+1$	1941.11/1941.12	1940.18*	−0.93/−194
$a_{10}/a_{10}+1$	1059.12/1060.13	1060.32	−1.20/+0.19	x_{18}	1967.06	1967.90	+0.84
b_{10}	1087.13	1086.57	−0.56	d_{19}	2012.19	2012.34	+0.15
x_9	1097.13	1097.35	+0.22	x_{19}	2082.15	2080.26	−1.80
w_{10}	1126.17	1127.05	−0.12	v_{20}	2110.21	2111.91	+1.70
y_{10}	1142.22	1140.97	+0.75	$z_{20}/z_{20}+1$	2126.20/2127.21	2126.43	+0.23/−0.78
$a_{12}/a_{12}+1$	1201.28/1202.29	1201.19	−0.09/−1.10	$a_{20}/a_{20}-1$	2170.30/2171.31	2170.72	+0.52/−0.59
x_{11}	1239.29	1239.60	+0.31	—COOH	2254.41	2254.06	−0.35
v_{12}	1267.34	1267.71	+0.37				

[a]An asterisk (*) indicate possible peak overlapping of fragment ions of similar mass value due to insufficient mass resolution of this time-of-flight instrument.

Table 6. Molecular weight determination of Lys–Arg-containing peptidoglycan fragments released by pronase E digestion from the bound form of Braun's lipoprotein, separated by reverse phase HPLC and identified by positive- and negative-ion PD TOF/MS; in the case of anhydro-peptidoglycan dimers and trimers all structures known to be present are listed (based on the work of Glauner and co-workers [17, 18]) because these isomers were not separated by this reverse phase HPLC system

HPLC fraction number R_t (min)	$[M+H]^+/[M+2H]^{2+}$ average m/z values		$[M-H]^-$ average m/z values		Common nomenclature (type of peptide side chain cross linkage; only with dimers and trimers) chemical structure of monomers/isomeric structures in case of anhydro dimers and trimers
	Calculated	Found	Calculated	Found	
1 (49.91)	1156.23	1156.3	1154.11	1154.3	Tri → Lys–Arg,
	578.62	578.7			GlcNAc → MurNAc → L–Ala → γ-D–Glu–L(D)–A2pm → ε-Lys ← Arg
2 (73.31)	2009.08	2008.4	2007.07	2007.2	Tri → Tri–Lys–Arg, (L–A_2pm → D–A_2pm)
	1005.05	1005.1			
3 (80.49)	2080.16	2080.1	2078.15	1986.0	Tetra → Tri–Lys–Arg, (D–Ala → D–A_2pm)
	1040.58	1041.0			
4 (90.16)	3004.10	3004.3	3002.07	3001.9	Tetra → Tetra → Tri–Lys–Arg, (D–Ala → D–A_2pm, D–Ala → D–A_2pm)
	1502.55	1052.6			
5 (102.77)	1989.05	1990.6	1987.04	1987.4	Tri → Tri–Lys–Arg (Anh), (L–A_2pm → D–A_2pm),
	995.03	995.1			Tri(Anh) → Tri–Lys–Arg and Tri → Tri–Lys–Arg(Anh)
6 (108.77)	2060.13	2060.0	2058.11	2056.8	Tetra → Tri–Lys–Arg(Anh), (D–Ala → D–A_2pm),
	1030.57	1030.7			Tetra(Anh) → Tri–Lys–Arg and Tetra → Tri–Lys–Arg(Anh)
7 (110.03)	2984.06	2984.2	2982.04	2981.2	Tetra → Tetra → Tri–Lys–Arg(Anh), (D–Ala → D–A_2pm, D–Ala → D–A_2pm),
	1492.53	1492.8			Tetra(Anh) → Tetra → Tri–Lys–Arg, Tetra → Tetra(Anh) → Tri–Lys–Arg and
					Tetra → Tetra → Tri–Lys–Arg(Anh)

The present work demonstrates a strategy for the rapid characterization of a strongly hydrophobic, water-insoluble lipoprotein by time-of-flight mass spectrometry. Significant structural information following appropriate selection of individual analyte solvent–support/matrix combinations for different classes of compounds was obtained by positive- and negative-ion PD TOF/MS and positive-ion MALDI TOF/MS. Whereas pure peptides (CNBr 2 and CNBr 3) and glycopeptides (Lys–Arg-containing peptidoglycan fragments) yielded satisfactory results by using the nitrocellulose adsorption technique as originally described by Jonsson et al. [25], other lipid-containing samples failed. For the chloroform-soluble lipid anchor we adapted our recently described sample preparation technique for lipids and glycolipids [20, 33–35] by using 3-(3-pyridyl) acrylic acid as matrix, thereby tolerating strongly lipophilic analyte solvents. Finally, for the water-insoluble free form of Braun's lipoprotein and for the cyanogen bromide cleavage peptide CNBr 1 (the lipopeptide), either nitrocellulose support or 9-anthroic acid or 3-(3-indolyl) acrylic acid matrix yielded satisfactory results by using formic acid: water:methanol:2-propanol (5:15:40:40) as analyte solvent. Additionally, for most compounds investigated (except for the free lipid anchor), MALDI TOF/MS gave better sensitivity and comparable data in terms of information content and mass spectrometric resolution if appropriate matrices were selected (sinapinic acid for the lipoprotein and 2,5-dihydroxybenzoic acid for glycopeptides).

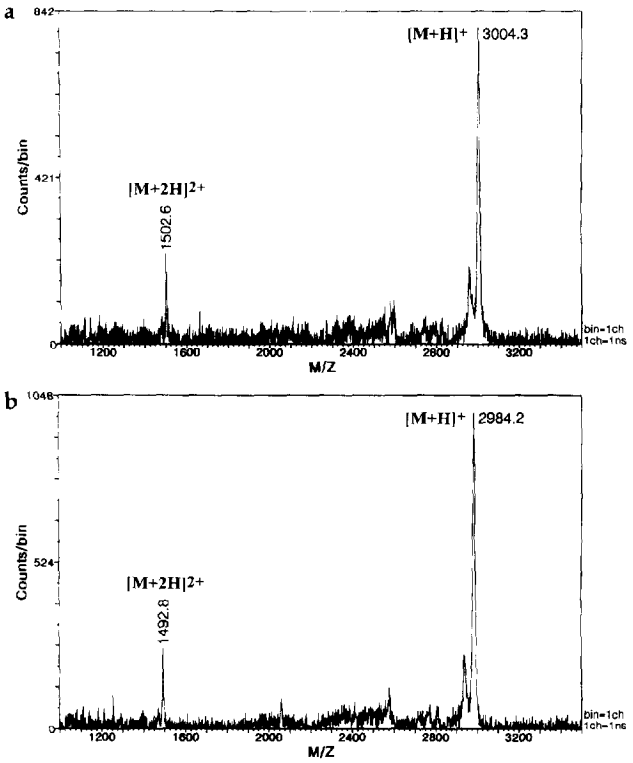


Figure 7. Positive-ion PD mass spectra of muramidase-released Lys–Arg-containing peptidoglycan trimers from the bound form of Braun's lipoprotein that represents (**a**) LC fraction 4, the trimer Terta–Tetra–Tri–Lys–Arg, and (**b**) LC fraction 7, the trimer Terta–Tetra–Tri–Lys–Arg (Anh), both adsorbed onto nitrocellulose support. For the corresponding LC fraction numbers 4 and 7, compare with Table 6.

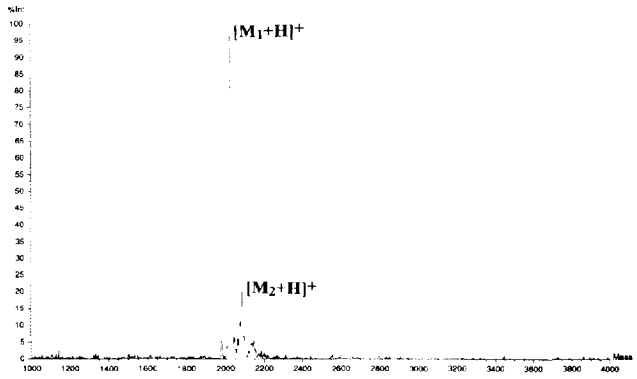


Figure 8. Positive-ion MALDI mass spectrum of the muramidase-released Lys–Arg-containing peptidoglycan dimer from the bound form of Braun's lipoprotein that represents LC fraction 2, the peptidoglycan dimer Tri–Tri–Lys–Arg denoted as $[M_1 + H]^+$. The small peak denoted as $[M_2 + H]^+$ corresponds to traces of the peptidoglycan dimer Tetra–Tri–Lys–Arg as an impurity of the next LC fraction. For the corresponding LC fraction numbers 2 and 3, compare with Table 6.

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