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Primary Structure of Cyanelle Peptidoglycan of *Cyanophora paradoxa*: a Prokaryotic Cell Wall as Part of an Organelle Envelope

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The peptidoglycan layer surrounding the photosynthetic organelles (cyanelles) of the protist *Cyanophora paradoxa* is thought to be a relic of their cyanobacterial ancestors. The separation of muropeptides by gel filtration and reverse-phase high-performance liquid chromatography revealed four different muropeptide monomers. A number of muropeptides were identical in retention behavior to muropeptides of *Escherichia coli*, while others had remarkably long retention times with respect to their sizes, as indicated by gel filtration. Molecular mass determination by plasma desorption and matrix-assisted laser desorption ionization mass spectrometry showed that these unusual muropeptides had molecular masses greater by 112 Da or a multiple thereof than those of ones common to both species. Fast atom bombardment-tandem mass spectrometry of these reduced muropeptide monomers allowed the localization of the modification to D-glutamic acid. High-resolution fast atom bombardment-mass spectrometry and amino acid analysis revealed *N*-acetylputrescine to be the substituent (E. Pittenauer, E. R. Schmid, G. Allmaier, B. Pfanzagl, W. Löffelhardt, C. Quintela, M. A. de Pedro, and W. Stanek, Biol. Mass Spectrom. 22:524–536, 1993). In addition to the 4 monomers already known, 8 dimers, 11 trimers, and 6 tetramers were characterized. An average glycan chain length of 51 disaccharide units was determined by the transfer of [U-^{14}C]galactose to the terminal *N*-acetylglucosamine residues of cyanelle peptidoglycan. The muropeptide pattern is discussed with respect to peptidoglycan biosynthesis and processing.

Among eukaryotes, peptidoglycan is found only in cyanelle-containing organisms. These consist of about 12 different species and have been grouped under the denomination glaucocystophytes (29). Recently, this has been corroborated by 18S rRNA-derived phylogenetic analysis (5). *Cyanophora paradoxa* is the only member of this group which can be grown easily; therefore, it is the best investigated one. The round cyanelles of this obligatorily photoautotrophic protist were originally regarded as endosymbiotic cyanobacteria for morphological reasons, such as thylakoid structure (13), carboxysomes (31), and a lysozyme-sensitive peptidoglycan sacculus (37). With the onset of molecular biological investigations of *C. paradoxa* (18, 32), it became clear that the cyanelle genome is smaller than the genomes of unicellular free-living cyanobacteria by a factor of about 25. Thus, cyanelles in fact represent the plastids of *C. paradoxa*, having retained the peptidoglycan layer which is crucial for division, as indicated by the inhibitory effects of β-lactam antibiotics (4, 29). The peptidoglycan layer is located between the organelle membrane and an outer membrane of unknown origin and has a thickness of about 7 nm (30).

Investigations of cyanelle peptidoglycan synthesis and degradation have shown that cyanelles apparently harbor a complete set of enzymes involved in these processes. Seven penicillin-binding proteins ranging from 110 to 35 kDa have been identified in the cyanelle envelope by labelling with a radioactive derivative of ampicillin (4). Indirect evidence has been obtained for periplasmic localization of DD- and LD-carboxypeptidases and DD-endopeptidase, enzymes capable of hydrolyzing defined bonds in peptidoglycan (36). The biosynthesis of the UDP–*N*-acetylmuramyl pentapeptide precursor of peptidoglycan has been shown to occur in the cyanelle stroma (36). The cyanelle genome (135.6 kb) of one of the two known isolates of *C. paradoxa*, strain LB555-UTEX, has been entirely sequenced (for a review, see reference 30). In addition to genes for the photosynthetic, transcription, and translation apparatus normally present on plastid DNAs, cyanelles encode more than 40 extra polypeptides, of which many are products of nuclear genes in higher plants. The enzymes for peptidoglycan synthesis and metabolism are presumably encoded in the nucleus, as the still-unidentified open reading frames on cyanelle DNA show no significant homology to the known enzymes for peptidoglycan synthesis and metabolism from other organisms.

A preliminary biochemical investigation of cyanelle peptidoglycan revealed amino acids and amino sugars typical of gram-negative bacteria (1). However, its unusual localization within an eukaryotic cell warrants a more thorough investigation. Osmotic protection certainly should not be the function of the peptidoglycan wall of a cell organelle in the environment of the cytoplasm. Plastids harbor approximately 1,000 different polypeptides, of which 900 have to be continuously imported from the cytoplasm where nucleus-encoded plastid proteins are synthesized as larger precursors. In contrast, bacteria secrete only a limited number of proteins into the medium. One could envisage that an organelle peptidoglycan would complicate the translocation of such a multitude of polypeptides

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TABLE 1. Cyanelle muropeptides[a]

Peak no.	Size fraction[b]	Avg $[M + H]^+$		Avg $[M - H]^-$		Amino acid ratio[c]		pH sensitivity of retention time (%)[d]	Proposed structure
		Observed	Calculated	Observed	Calculated	A/G	A/D		
1	IV	871.7	871.9	869.8	869.9	ND[e]	ND	20–30	Tri
2	IV	943.0	942.9	940.9	940.9	ND	ND	20–30	Tet
3	III	1,795.4	1,795.8	1,793.1	1,793.8	ND	ND	20–30	Tet-Tri
4	IV	983.9	984.0	981.4	982.0	0.98	1.38	0–10	Tri(NAP)
5	III	1,867.5	1,866.9	ND	ND	ND	ND	20–30	Tet-Tet
6	IV	1,055.1	1,055.1	1,053.0	1,053.1	1.96	2.37	0–10	Tet(NAP)
7	II	2,719.2	2,719.7	ND	ND	1.68	1.64	20–30	Tet-Tet-Tri
8	III	1,907.6	1,908.0	1,905.2	1,905.8	1.54	1.73	10–20	Tet-Tri(NAP)[f]
9	III	1,907.7	1,908.0	ND	ND	1.48	1.56	10–20	Tet(NAP)-Tri[f]
10	III	1,979.7	1,979.0	ND	ND	1.87	2.37	10–20	Tet-Tet(NAP)[f]
11	III	1,979.3	1,979.0	ND	ND	1.96	2.31	10–20	Tet(NAP)-Tet[f]
12	II	2,831.5	2,831.9	ND	ND	1.58	1.98	20–30	Tet-Tet(NAP)-Tri[f]
13	II	2,831.1	2,831.9	ND	ND	1.58	2.24	20–30	Tet(NAP)-Tet-Tri[f]
14	III	2,020.1	2,020.1	ND	ND	1.33	1.80	0–10	Tet(NAP)-Tri(NAP)
15	II	2,944.4	2,944.1	ND	ND	1.63	2.55	10–20	Tet(NAP)-Tet(NAP)-Tri[f]
16	III	2,091.0	2,091.2	2,088.8	2,089.2	2.01	2.12	0–10	Tet(NAP)-Tet(NAP)
17	II	2,701.2	2,699.7	ND	ND	ND	ND	ND	Tet-Tet-Tri-Anh
18	II	2,943.0	2,944.1	ND	ND	ND	ND	ND	Tet-Tet(NAP)-Tri(NAP)[f]
19	II	2,942.8	2,944.1	ND	ND	ND	ND	ND	Tet(NAP)-Tet-Tri(NAP)[f]
20	II	3,014.1	3,015.1	ND	ND	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tet[f]
21	II	2,810.9	2,810.0	ND	ND	ND	ND	ND	Tet(NAP)-Tet-Tri-Anh[f]
22	II	3,055.5	3,056.2	ND	ND	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tri(NAP)
23	II	3,125.4	3,127.3	ND	ND	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tet(NAP)
24	I	3,794.4[g]	3,755.6	3,754.3	3,753.6	ND	ND	ND	Tet(NAP)-Tet-Tet-Tri[f]
25	I	3,888.3[h]	3,867.9	3,868.1	3,865.9	ND	ND	ND	Tet(NAP)-Tet-Tet(NAP)-Tri[f]
26	I	3,868.2	3,867.9	3,869.8	3,865.9	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tet-Tri
27	I	3,979.9	3,980.1	3,979.0	3,978.1	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tet(NAP)-Tri[f]
28	I	4,093.0	4,092.3	4,091.2	4,090.3	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tet(NAP)-Tri(NAP)
29	I	4,165.3	4,163.3	4,161.6	4,161.3	ND	ND	ND	Tet(NAP)-Tet(NAP)-Tet(NAP)-Tet(NAP)

[a] Peak numbers correspond to the numbers given in Fig. 1. The molecular weights of muropeptides were determined by PDMS (peaks 1 to 23) or MALDIMS (peaks 24 to 29).

[b] As shown in Fig. 2.

[c] A, alanine; G, glutamic acid; D, diaminopimelic acid.

[d] The sensitivity of the retention time to variations in the pH of the elution buffer was determined by comparing the retention times at pH 5.1 and pH 5.5 at room temperature. Retention time differences were calculated as percentages of the retention time at pH 5.1. They were grouped into three sensitivity classes (0 to 10, 10 to 20, and 20 to 30%).

[e] ND, not determined.

[f] For this muropeptide, it is not known which of the subunits carries the NAP residue. Its assignment to a particular subunit is based on the assumption that NAP is concentrated on tetrapeptide side chains in cross-linked muropeptides, as it is in muropeptide monomers.

[g] $[M + K]^+$; calculated molecular weight of the potassium adduct, 3,793.7.

[h] $[M + Na]^+$; calculated molecular weight of the sodium adduct, 3,889.8.

unless some modifications had occurred in the course of evolution. In this paper, we present detailed data on the fine structure of the cyanelle peptidoglycan sacculus.

MATERIALS AND METHODS

Preparation of cyanelle peptidoglycan. Both strains of *C. paradoxa* (LB555-UTEX and 1555) were grown as previously described (6). In general, cells were harvested in the exponential growth phase. Cyanelle peptidoglycan was prepared from whole cells by a protocol developed for *Escherichia coli* (19). α-Amylase from *Bacillus subtilis* and pronase E from *Streptomyces griseus* were purchased from Boehringer (Mannheim, Germany). Peptidoglycan was digested overnight at 37°C with 20 μg of *Chalaropsis* muramidase (17) per ml in 50 mM potassium phosphate buffer (pH 4.9) to produce muropeptides. Unsolubilized material, which contained less than 4% of total diaminopimelic acid, was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was kept in a boiling water bath for 5 min to inactivate muramidase. Insoluble material was again removed by centrifugation. Peptidoglycan of *Synechocystis* sp. strain PCC 6714 was the gift of U. Jürgens (Freiburg, Germany).

Reduction and β-elimination of muropeptides. For the reduction at C-1 of *N*-acetylmuramic acid, muropeptides were incubated with 8 mg of sodium borohydride per ml in 25 mM sodium borate buffer (pH 8) for 20 min. β-Elimination (42) was achieved by the incubation of unreduced muropeptides in ammonium hydroxide (pH 11) for 8 h at 37°C. The reaction was stopped either by adjustment of the pH to 4.5 with *ortho*-phosphoric acid or by evaporation to dryness.

Size fractionation of muropeptides with Biogel P6. Unreduced cyanelle muropeptides were applied to a Biogel P6 column (700 by 16 mm; Bio-Rad, Hercules, Calif.) and eluted with 100 mM LiCl in 20 mM potassium phosphate buffer (pH 6; flow rate, 10 ml/h). Fractions of 0.63 ml were collected, and the A_{214} was determined. Fractions belonging to the same peak were pooled, desalted via Biogel P6, reduced, and subjected to high-performance liquid chromatography (HPLC) for identification or preparative purposes. The results are listed in Table 1. The diaminopimelic acid contents of individual molecular weight fractions were determined from a volume of 600 μl after concentration under a vacuum and hydrolysis in 6 M HCl at 110°C for 18 h in a total volume of 200 μl.

HPLC of muropeptides. For the separation of muropeptides, the HPLC method developed by Glauner et al. (14, 15) (Hypersil octyldecyl silane column [250 by 4.6 mm]; particle size, 5 μm; detection at 214 nm). Reduced muropeptides were eluted with a linear gradient from 0 to 20% of methanol in 50 mM potassium phosphate buffer (pH 5.1) at a flow rate of 0.5 ml/min. The gradient was started 7 min after injection of the sample and reached final conditions 157 min later. Then isocratic conditions were kept for another 35 min. Dimeric muropeptides and major trimers collected from this gradient after injection of total cyanelle muropeptides were further purified by a second run at pH 4.65 under isocratic conditions, with the percentage of methanol adjusted for optimal separation in each case. Unreduced muropeptide monomers and N^1-lactylpeptides were eluted at pH 3.6 by using the same methanol gradient. At 40 min after injection of the sample, the flow rate was changed from 0.5 to 1 ml/min. All HPLC separations were carried out at room temperature.

^{252}Cf-plasma desorption mass spectrometry (PDMS), matrix-assisted laser desorption-ionization mass spectrometry (MALDIMS), and fast atom bombardment (FAB)-tandem mass spectrometry. The muropeptides collected from the

methanol gradient in phosphate buffer were desalted for mass spectrometry and thereby further purified, as described elsewhere (10, 35).

PDMS measurements were performed on a time-of-flight PDMS (BioIon AB, Uppsala, Sweden) with a flight tube length of 141 mm. Acceleration voltages were 17 and 15 kV in the positive and negative ionization modes, respectively. Spectra measured in the positive and negative ionization modes were accumulated for 5 and 10 million fission events, respectively. Samples were prepared by the so-called nitrocellulose sandwich technique described elsewhere (2) to remove alkali contaminants and thus improve protonated molecular ion yield.

MALDIMS measurements were performed in the positive-ion linear mode and negative-ion reflector mode on a Kompact MALDI-III (Kratos Analytical, Manchester, United Kingdom) laser desorption time-of-flight instrument equipped with a nitrogen UV laser (λ, 337 nm; 3-ns pulse width). Mass spectra were obtained by signal averaging of 15 to 80 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). Ions were accelerated to a final potential of +20 or −20 kV. The conversion of flight time to mass/charge was achieved by calibration using the $[M + H]^+$ or $[M - H]^-$ peak of the matrix and the $[M + H]^+$ or $[M - H]^-$ peaks of standard peptides (angiotensin I, arginine-8-vasopressin, atrial natriuretic peptide, hirudin, insulin, oxytocin, and somatostatin). Mass assignments for the peptidoglycan oligomers were derived from the centroided tops of the ion signals. Sample preparation was done by the recently reported procedure with a 2,5-dihydroxybenzoic acid matrix (41). The sample-matrix mixtures were applied in the center of the disposable sample probe, and solvents were removed slowly with the system apparatus, forming large crystals before insertion into the ion source. The peptidoglycan oligomers did not yield any improved mass spectra by applying the α-cyano-4-hydroxycinnamic acid or sinapinic acid matrix.

Positive-ion FAB mass spectra were obtained with a Finnigan MAT H-SQ 30 hybrid tandem mass spectrometer (Finnigan MAT, Bremen, Germany) fitted with a saddle-field FAB gun (Ion Tech, Teddington, United Kingdom) operating at 8 to 9.5 keV with xenon atoms and a 0.4-mA tube current. Glycerol containing 0.1% trifluoroacetic acid was used as a liquid matrix. The ^{12}C species of the $[M + H]^+$ isotopic ion cluster were selected MS1 (double focusing analyzer). Collision-induced dissociation spectra were obtained by operating the first quadrupole as collision region at a precursor ion signal reduction approximately 50% with a collision energy of 45 eV (laboratory frame of reference). Argon was used as the collision gas. Accurate mass measurements were obtained at a resolving power of approximately 5,000 (10% valley definition) with a sector instrument type 95 (Finnigan MAT).

Amino acid analysis of isolated muropeptides and dinitrophenylation. For amino acid analysis, desalted reduced muropeptides were hydrolyzed with 6 M HCl for 18 h at 110°C and vacuum dried. Derivatization with *ortho*-phthaldialdehyde and separation of the derivatives by HPLC were carried out as described previously (9). Because of its long retention time, putrescine was not detected. A modification of the procedure suitable to detect putrescine has been described elsewhere (35). Dinitrophenylation with subsequent hydrolysis was carried out as previously described (9). Hydrolyzed samples were submitted to amino acid analysis.

Determination of the percentage of modified peptide side chains. Muropeptides obtained from 3 liters of culture were hydrolyzed in 6 M HCl at 110°C for 18 h in a total volume of 200 μl. After being dried under a vacuum, samples were resuspended in 20 μl of water and the pH was adjusted to 9 to 10 with NaOH. Amino groups were dabsylated essentially by the method of Chang et al. (11). Samples (0.25 to 3 μl) were diluted with water to a total volume of 10 μl. After the addition of 10 μl of 0.5 M sodium borate buffer (pH 10) and 200 μl of 4-dimethylaminoazobenzene-4′-sulfonyl chloride (2 nmol/μl in acetone), the mixture was heated for 10 min at 70°C. Acetone was then evaporated under a vacuum, and 20 μl of acetonitrile and 160 μl of 50 mM potassium phosphate buffer (pH 6.2) were added. Half of the sample was subjected to HPLC at room temperature (Hypersil octyldecyl silane column [250 by 4.6 mm]; particle size, 5 μm) with a gradient from 17 to 70% acetonitrile in 50 mM phosphate buffer (pH 6.2) and a flow rate of 1 ml/min. For the first 21 min, the concentration of acetonitrile was increased by 0.7% per min; then the increase was accelerated to 1.9% per min. Dabsylated compounds were detected by absorption at 436 nm. For quantification, a standard solution containing known amounts of glutamic acid and putrescine was used.

Determination of glycan chain length. The average length of glycan chains was determined by the method of Schindler et al. (38). A peptidoglycan sample containing about 50 nmol diaminopimelic acid was incubated in 50 mM Tris buffer (pH 8.4)–20 mM $MnCl_2$ with 0.37 μCi of UDP-D-[U-^{14}C]galactose (268 μCi/μmol; Du Pont New England Nuclear, Boston, Mass.), 32.3 nmol UDP-galactose, and 0.25 U of galactosyltransferase from cow milk (Sigma, St. Louis, Mo.) in a total volume of 100 μl. After incubation for 2 h at 30°C, peptidoglycan was washed several times with water by centrifugation at 10,000 × *g* for 10 min until no radioactivity could be detected in the supernatant. Peptidoglycan was then digested with *Chalaropsis* muramidase as described above, and the radioactivity of the muropeptide solution was determined by using Ecolume (ICN Biomedicals, Irvine, Calif.) as a scintillation cocktail. The muropeptide solution obtained from a control incubated without galactosyltransferase did not contain any radioactivity above the background level. For the determination of the diaminopimelic acid content, two aliquots of muropeptide solution were hydrolyzed in 6 M HCl at 110°C for 18 h.

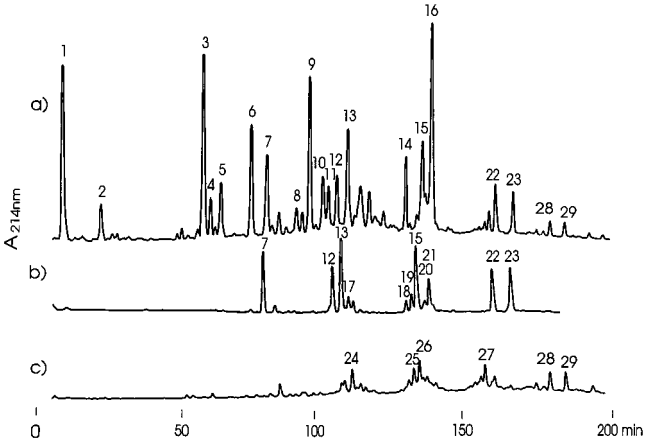

FIG. 1. Reverse-phase HPLC of the following reduced cyanelle muropeptides at pH 5.1: total cyanelle muropeptides (a) and fractions II (muropeptide trimers) (b) and I (muropeptide tetramers and higher oligomers) (c) (see Fig. 2). Structures for peak numbers are given in Table 1.

Determination of diaminopimelic acid. Hydrolyzed muropeptide samples were dried under a vacuum and resuspended in 50% acetic acid in water. Ninhydrin reagent (250 mg of ninhydrin, 6 ml of acetic acid, 4 ml of 0.6 M H_3PO_4) (0.5 ml) was added, and samples were heated in a boiling water bath for 5 min. Then the absorption at 436 nm was determined and compared with standards of 0 to 20 μg of diaminopimelic acid (45).

RESULTS

HPLC of cyanelle muropeptides. The muropeptide patterns obtained after HPLC for the two isolates of *C. paradoxa* (LB555-UTEX and 1555) were identical. All further experiments were conducted with peptidoglycan from strain LB555-UTEX. In comparing the HPLC elution patterns of cyanelle and *E. coli* muropeptides, the most remarkable differences are the small number of muropeptides with short retention times and the abundance of high-intensity peaks that elute later than any major muropeptide found in *E. coli*. The sensitivity of the retention times of muropeptides to variations in the pH of the elution buffer was used to test cyanelle and *E. coli* muropeptides with equal retention times for coelution at various pH values. This procedure led to the preliminary identification of cyanelle muropeptide peaks 1, 2, 3, 5, and 7 (Fig. 1a) as the disaccharide-tripeptide *N*-acetylglucosaminyl(GlcNAc)-*N*-acetylmuramyl(MurNAc)-L-Ala-γ-D-Glu-*m*-Dap (Tri), the disaccharide-tetrapeptide GlcNAc-MurNAc-L-Ala-γ-D-Glu-*m*-Dap-D-Ala (Tet), the bis-disaccharide-tetra-tripeptide GlcNAc-MurNAc-L-Ala-γ-D-Glu-*m*-Dap-D-Ala→*m*-Dap-γ-D-Glu-L-Ala-MurNAc-GlcNAc (Tet-Tri), the analogous bis-disaccharide-tetra-tetrapeptide (Tet-Tet), and tris-disaccharide-tetra-tetra-tripeptide (Tet-Tet-Tri), respectively.

Search for covalently bound protein. The omission of pronase treatment during peptidoglycan preparation did not alter the muropeptide pattern obtained after HPLC, showing that no cyanelle muropeptide contains residual amino acids indicating covalently bound lipoprotein. Likewise, the Bradford assay (7) did not detect any protein in muropeptide solutions prepared without pronase treatment of peptidoglycan.

Size exclusion chromatography and HPLC of the molecular weight fractions. The separation of muropeptides by size exclusion chromatography with Biogel P6 is shown in Fig. 2. HPLC analysis of the materials in peaks I, II, III, and IV allowed the assignment of unidentified muropeptides to mo-

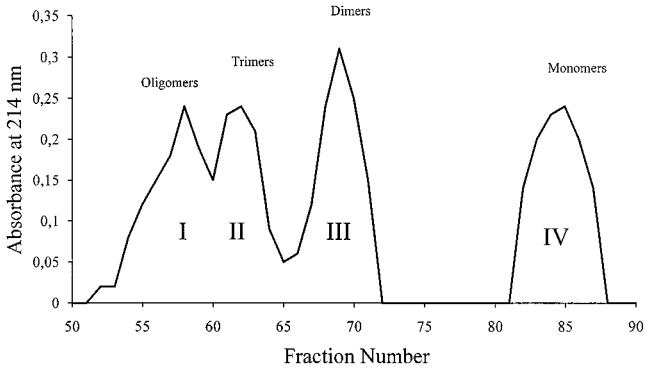

FIG. 2. Size fractionation pattern of unreduced cyanelle muropeptides on a Biogel P6 column. Fractions belonging to the same peak were pooled, reduced, and subjected to HPLC for identification. The results are listed in Table 1.

lecular weight groups (monomers, dimers, trimers, and higher oligomers [Table 1]), confirming the preliminary identifications of cyanelle muropeptides also present in *E. coli* peptidoglycan. Considering that peaks 4 and 6 (Fig. 1a) are monomeric muropeptides, they have remarkably long retention times which vary only slightly with changes in the pH of the elution buffer (Table 1). Since the pH sensitivity of retention times below pH 7 is mediated by dissociation of free carboxyl groups, a rather hydrophobic substituent bound to one of the carboxyl groups not engaged in peptide bonds was considered a possible explanation for this behavior.

Repeated muramidase digestions of fractions I and II did not alter their HPLC profiles, proving that muramidase digestion had been complete. Amino acid analysis of fraction I, which elutes close to the exclusion volume of the column, showed that it contained only peptidoglycan amino acids.

Mild treatment with NaOH and β-elimination of muropeptides. Overnight treatment of cyanelle peptidoglycan with 0.1 M NaOH at 37°C did not alter the muropeptide pattern obtained after muramidase digestion and HPLC. This is in accordance with the high lysozyme sensitivity of cyanelle peptidoglycan and excludes the possibility that O acetylation of muramic acid is the reason for the long retention times of cyanelle muropeptides. The N^1-lactylpeptides produced by β-elimination of the unreduced forms of muropeptides 4 and 6 had much longer retention times than those of the N^1-lactylpeptides from disaccharide-tripeptide and disaccharide-tetrapeptide (data not shown). Therefore, the hydrophobic modification was localized to the peptide part of muropeptides.

Amino acid analysis and dinitrophenylation. No amino acids other than alanine, diaminopimelic acid, and glutamic acid were detected in the hydrolysate of novel muropeptides. Their proportions are given in Table 1. The relative amount of diaminopimelic acid susceptible to dinitrophenylation (data not shown) in each case was in accordance with the assignment as a monomeric or cross-linked oligomeric muropeptide based on size exclusion chromatography and PDMS (see below).

PDMS and MALDIMS. The major peaks obtained after HPLC of total cyanelle peptidoglycan were subjected to PDMS. The results are listed in Table 1. Selected mass spectra are shown in Fig. 3. The already anticipated identities of peaks 1, 2, 3, 5, and 7 (Fig. 1) as the unmodified muropeptides Tri, Tet, Tet-Tri, Tet-Tet, and Tet-Tet-Tri, respectively, were con-

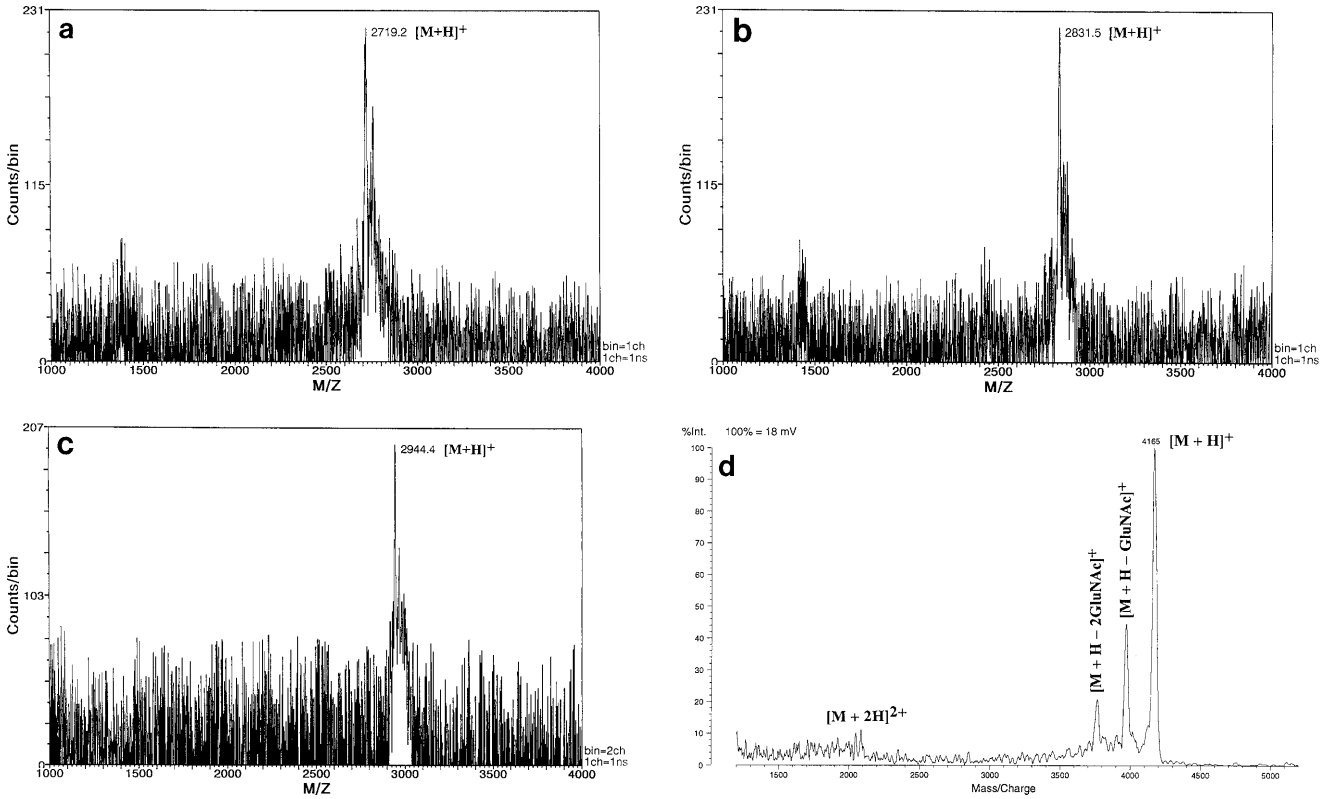

FIG. 3. Positive-ion PDMS of reduced oligomeric cyanelle muropeptides. (a) Tris-disaccharide-tetra-tetra-tripeptide (peak 7 [Fig. 1a]); (b) a monosubstituted tris-disaccharide-tetra-tetra-tripeptide (peak 12 [Fig. 1a]); (c) a disubstituted tris-disaccharide-tetra-tetra-tripeptide (peak 15 [Fig. 1a]); (d) positive-ion MALDIMS of the reduced tetrameric cyanelle muropeptide tetrasubstituted tetrakis-disaccharide-tetra-tetra-tetrapeptide (peak 29 [Fig. 1c]).

firmed. A second group of muropeptides consisting of peaks 4, 6, and 8 to 13 showed a consistent mass shift of 112 Da compared with those of unmodified muropeptides. Peaks 14, 15, and 16, the latest eluting peaks isolated from total peptidoglycan, showed twice this shift, i.e., an increase in mass of 224 Da. This was a strong indication that a substituent with a molecular mass of 112 Da bound to part of the muropeptide subunits of cyanelle peptidoglycan. In addition, minor trimers and the most abundant tetramers were isolated from fractions II and I, respectively, after size exclusion chromatography of muropeptides obtained from large-scale preparations of cyanelle peptidoglycan. The molecular weights of these minor trimers were determined by PDMS, whereas tetramers were subjected to the more sensitive MALDIMS because of the small quantities present (Table 1). Among the minor trimers eluting close to peaks 14 to 16, two isomers of peak 15, as well as doubly substituted Tet-Tet-Tet (peak 20) and one muropeptide with a molecular weight corresponding to that of monosubstituted Tet-Tet-Tri with 1,6-anhydro-*N*-acetylmuramic acid (Tet-Tet-Tri-Anh), were found. A muropeptide with the molecular weight of unsubstituted Tet-Tet-Tri-Anh was also found among the minor trimers, eluting close to the monosubstituted Tet-Tet-Tri isomers. As expected from their long retention times, peaks 22 and 23 turned out to be Tet-Tet-Tri and Tet-Tet-Tet, respectively, carrying three substituents. The six tetramers analyzed by MALDIMS had one to four substituents. Peak 29, fully substituted Tet-Tet-Tet-Tet, represents the largest muropeptide measured by MALDIMS (Fig. 3d). A strong dependence of retention times on the number of substituents was observed for dimers, trimers, and tetramers without Anh, leading to the clustered elution of muropeptides with the same number of substituents (Fig. 1b and c). By positive-ion PDMS of the modified monomeric N^1-lactylpeptides, glutamic acid was shown to be γ linked to diaminopimelic acid (35).

FAB-mass spectrometry. High-resolution FAB measurement of the modified N^1-lactyltetrapeptide yielded an $[M + H]^+$ ion with a molecular weight of 646.3415, fitting best to an elemental composition of $C_{27}H_{48}N_7O_{11}$ (calculated M_r, 646.3412). Thus, the modifying compound (deduced elemental composition, $C_6H_{14}N_2O$) was expected to be *N*-acetylputrescine (NAP) (*N*-acetyl-1,4-diaminobutane; M_r, 130), which upon hydrolysis should yield putrescine. Putrescine was indeed detected in the hydrolysate of muropeptide 6 after the procedure of amino acid analysis had been adapted for this purpose as previously described (35). The fragmentation pattern of the reduced modified disaccharide-tetrapeptide (peak 6 in Fig. 1a) obtained by FAB-mass spectrometry unambiguously showed that the modification was bound to the glutamic acid residue (Fig. 4). This was indicated by the mass difference of 241 units (that of Glu + 112 units) between *m/z* 503 and *m/z* 262.

Glycan chain length and chain ends. The average length of the glycan chains of cyanelle peptidoglycan was determined by the addition of D-[U-^{14}C]galactose to the C-4 of terminal *N*-acetylglucosamine moieties with galactosyltransferase and subsequent muramidase hydrolysis. For *E. coli* (38), this method yielded an average chain length about 40% higher than that determined by the separation of individual glycan strands by HPLC (16). An amount of cyanelle peptidoglycan with a diaminopimelic acid content of 48.5 nmol (mean of two determinations) yielded a muropeptide solution containing 23,257 dpm of D-[U-^{14}C]galactose (11 nCi/nmol). From this, an average glycan chain length of 50.8 ± 2.6 disaccharide units was calculated. Treatment with larger-than-usual amounts of α-amylase during the preparation of cyanelle peptidoglycan did not alter the average glycan chain length, meaning that the α-amylase preparation did not contain muramidase. To control

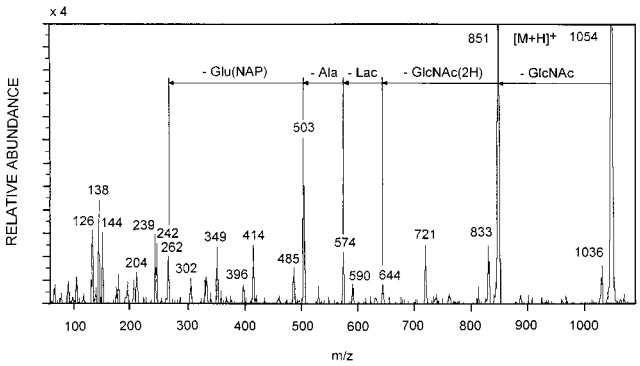

FIG. 4. Positive-ion low-energy collision-induced dissociation spectrum of the $[M + H]^+$ ion (*m/z* = 1,054.6, generated by FAB) of the reduced modified disaccharide-tetrapeptide (peak 6 [Fig. 1a]). The fragmentation pattern indicates that the NAP residue is located at the glutamyl moiety. GlcNAc, *N*-acetylglucosamine; GlcNAc(2H), reduced *N*-acetylglucosamine; Lac, lactic acid; Glu(NAP), glutamic acid amidated with NAP.

their identities, galactosylated cyanelle muropeptides were subjected to β-elimination to obtain trisaccharides which were compared with trisaccharides from galactosylated dimeric muropeptides by descending paper chromatography in butanol-acetic acid-water (3:1:1 [vol/vol/vol]) on Whatman 3MM paper and had the same R_f (0.2), while galactose had an R_f of 0.1. The molecular weights of peaks 17 and 21 (Fig. 1 and Table 1) determined by PDMS potentially correspond to Tet-Tet-Tri-Anh and Tet(NAP)-Tet-Tri-Anh, respectively, with one of their three *N*-acetylmuramic acid residues in the 1,6-anhydro form. These structures would also be consistent with their retention times by HPLC (15). A comparison of the areas of peaks 21 and 13 indicates that about one-fourth of all Tet (NAP)-Tet-Tri muropeptides have one Anh. In contrast, no anhydro forms of monomeric and dimeric muropeptides were detected. This suggests higher levels of cross-linkage at chain ends, as has been found in *E. coli* (15). The incubation of whole cyanelle peptidoglycan at a high pH, which causes β-elimination of lactylpeptides at reducing chain ends but not at anhydro ends, did not noticeably change the muropeptide pattern obtained after muramidase digestion and HPLC, giving no evidence for reducing ends of glycan chains. However, a mean chain length of about 50 disaccharide units might well render the quantity of muropeptides derived from chain ends (2%) difficult to detect by the methods applied. Alternative methods for the detection of reducing compounds were not applicable because of reducing impurities in the peptidoglycan preparation.

Variation of the muropeptide pattern with the state of growth. Compared with cells in the exponential-growth phase, cells approaching the stationary phase because of nitrogen limitation or light deprivation for one generation show a significant reduction in the amounts of Tet, Tet(NAP), Tri(NAP), and Tet(NAP)-Tet(NAP). In contrast, there is a slight increase in the percentage of Tri and a pronounced increase in the percentage of Tet-Tri (Table 2). These changes cannot be attributed to the different composition of a newly synthesized peptidoglycan, as it also occurs when cells are treated with ampicillin (5 μg/ml) and kept in darkness. Ampicillin can enter *C. paradoxa* and binds with high affinity to the penicillin-binding proteins of cyanelles, preventing cyanelle growth and division (4, 29). Although cyanelles remain osmotically stable, with their autolysis system apparently tightly controlled, this growth inhibition eventually leads to cell death in growing *C. paradoxa*

TABLE 2. Relative amounts of individual muropeptide monomers and dimers in exponentially growing cultures and cultures at the onset of stationary growth

Peak no.	% of total monomers or dimers in culture (%)[a]		Proposed structure
	Exponential growth (range)	Stationary growth (range)	
1	51.0 (41.1–63.9)	68.8 (59.9–81.5)	Tri
2	11.1 (6.1–22.2)	3.5 (0.0–9.0)	Tet
4	13.4 (9.6–17.9)	11.7 (6.3–14.3)	Tri(NAP)
6	24.4 (13.9–29.7)	15.9 (10.0–13.4)	Tet(NAP)
3	20.1 (20.0–20.2)	26.9 (25.1–28.7)	Tet-Tri
5	2.2 (1.6–2.8)	2.8 (2.7–2.9)	Tet-Tet
8	3.3 (3.2–3.4)	4.5 (4.2–4.8)	Tet-Tri(NAP)
9	17.9 (16.7–19.1)	21.4 (21.3–21.5)	Tet(NAP)-Tri[b]
10	4.6 (4.5–4.7)	5.9 (5.0–6.8)	Tet-Tet(NAP)[b]
11	2.4 (1.9–3.3)	2.2 (2.1–2.3)	Tet(NAP)-Tet[b]
14	16.8 (16.7–16.9)	14.9 (13.8–16.0)	Tet(NAP)-Tri(NAP)
16	32.7 (32.3–33.1)	21.3 (20.4–22.2)	Tet(NAP)-Tet(NAP)

[a] Relative amounts of individual muropeptide monomers (peaks 1, 2, 4, and 6) and dimers (peaks 3, 5, 8 through 11, 14, and 16) in percentages of the total amounts of monomers and dimers, respectively, in exponentially growing cultures and cultures at the onset of stationary growth. Data are averages of six experiments in the case of monomers and two experiments in the case of dimers. The percentages of muropeptide subunits (disaccharide and peptide side chain) participating in the different kinds of muropeptides were calculated from the peak area percentages (A_{214}) of the muropeptides. Since the individual extinction coefficients are unknown, the approximation developed by Glauner (14) for the muropeptides of *E. coli* was used for the conversion of UV data into molar percentages. For this calculation, it was assumed that the contribution of one NAP residue equals that of two amide bonds.

[b] See Table 1, footnote *f*.

cells, not in stationary cells. Keeping the cells in darkness for 1 more day does not lead to further changes in muropeptide composition.

As expected from this change in muropeptide pattern with the state of growth, the percentage of peptide side chains modified with NAP, as determined by amino acid analysis, was much higher for exponentially growing cells (60% ± 6%) than for cells which had been kept in darkness for 24 h with or without ampicillin (40% ± 3%). These values are in good agreement with the estimates made from the peak areas of different muropeptides after HPLC (data not shown).

The cross-linkages of peptide side chains (14) for one exponential-growth culture and one culture approaching stationary phase were calculated from double determinations of the diaminopimelic acid contents of the four molecular weight fractions (Fig. 2) obtained after size exclusion chromatography of muropeptides. In each case, a good correlation between the diaminopimelic acid content and A_{214} was obtained. In the exponentially growing culture, 12.7% ± 1.3% of total diaminopimelic acid was found in the monomer, 44.8% ± 0.1% was found in the dimer, 23.0% ± 0.5% was found in the trimer, and 19.5% ± 1.3% was found in the higher oligomer fraction. In the culture kept in darkness, 14.2% ± 0.7% of total diaminopimelic acid was found in the monomer, 35.0% ± 0.8% was found in the dimer, 28.0% ± 0.4% was found in the trimer, and 22.7% ± 0.1% was found in the higher oligomer fraction. To simplify the calculation, the whole diaminopimelic acid content of fraction I was attributed to tetrameric muropeptides. The error resulting from this assumption can be expected to be less than 1%. The cross-linkage was found not to change significantly between exponentially growing cells (52.4% ± 1.7%) and cells approaching stationary phase (53.2% ± 0.8%).

DISCUSSION

A detailed analysis of cyanelle peptidoglycan by HPLC and mass spectrometry revealed a structure of moderate complexity. The average length of glycan chains was determined to be 50.8 ± 2.6 disaccharide units. No O acetylation of sugar residues was detected. The peptide side chains are composed of alanine, γ-linked D-glutamic acid (35), and *m*-diaminopimelic acid, and cross bridges are formed between D-alanine and *m*-diaminopimelic acid. The D-alanine residues in positions 4 and 5 not involved in cross-linkage are apparently removed to a large extent, resulting in a lack of pentapeptide side chains and a large amount of tripeptide side chains. Disaccharide-tripeptide might also be incorporated directly into cyanelle peptidoglycan from lipid-linked N-acetylmuramyl-tripeptide, as has been shown to occur in *E. coli* (44). The significant characteristic of cyanelle muropeptide composition is the partial amidation of the 1-carboxyl group of glutamic acid with NAP. Amidation of this carboxyl group with ammonia is frequently found in gram-positive bacteria as well as gram-negative bacteria (39). In some gram-positive species, alanine, glycine, and glycinamide are found in this position. In anaerobic gram-negative bacteria (*Selenomonas*, *Veillonella*, and other species) phylogenetically grouped with gram-positive bacteria, cadaverine and putrescine have been shown to occupy this position (23–28, 40). To our knowledge, NAP has not previously been found as a constituent of peptidoglycan. In cyanelle peptidoglycan, 60% ± 6% of all muropeptide subunits are modified with NAP in exponentially growing cells and 40% ± 3% of all such subunits in cells approaching stationary phase are modified with NAP. Even after 3 days of nitrogen starvation, about 30% of all muropeptide subunits, as estimated from HPLC patterns of muropeptides, remain modified (data not shown). The distribution of NAP among the various types of muropeptides is not statistical. In monomeric muropeptides, NAP is clearly concentrated on tetrapeptide side chains. Incorporation might already occur at the precursor level, as has been shown for cadaverine in *Selenomonas ruminantium* (28). With L-Ala-γ-D-Glu(NAP)-*m*-DAP-D-Ala-D-Ala as the donor side chain for the cross-linking reaction, the main acceptors would be L-Ala-γ-D-Glu(NAP)-*m*-DAP-D-Ala side chains [Tet (NAP)], giving rise to the observed large quantity of Tet (NAP)-Tet(NAP). This could result from a preference for Tet (NAP) in the cross-linking reaction or from an increased percentage of Tet(NAP) in a putative growth zone. The change in the muropeptide pattern observed when peptidoglycan synthesis slows down or halts suggests that an LD-carboxypeptidase and an enzyme cleaving off NAP continue their actions on the peptidoglycan sacculus for a while after the incorporation of new peptidoglycan has ceased. A similar shift of tetrapeptide side chains to tripeptide side chains has been observed in *E. coli* (15, 43).

The peptidoglycan of the second known isolate of *C. paradoxa*, strain 1555, which differs from that of strain LB555-UTEX in size and the restriction pattern of cyanelle DNA (8) and in the organization of its nuclear genes coding for rRNA (3), was also examined by HPLC. No differences in the muropeptide compositions of these two strains were observed. Nor did previous investigations of penicillin-binding proteins show any differences between these two strains in the numbers or molecular weights of these proteins (33).

With respect to the proposed endosymbiotic origin of cyanelles from cyanobacteria, the peptidoglycan (containing covalently linked polysaccharides) of *Synechocystis* sp. strain PCC 6714, a cyanobacterium similar in shape to a cyanelle, was also subjected to preliminary analysis by HPLC and PDMS (data

not shown). The peptidoglycan composition and structure of this organism have already been examined by other methods (21). In agreement with earlier results, muropeptides containing NAP were not detected in *Synechocystis* peptidoglycan. As in cyanelle muropeptides not modified with NAP, terminal D-alanine residues not participating in cross-links are almost completely absent from *Synechocystis* peptidoglycan. Its cross-linkage seems to be much higher than that of cyanelle peptidoglycan, as evidenced by the appearance of muropeptide pentamers, hexamers, and heptamers (34) in accordance with the greater thickness of the cyanobacterial peptidoglycan layer. In neither *Synechocystis* (21) nor cyanelle peptidoglycan has any covalently linked protein been detected, while polysaccharide is bound to *Synechocystis* peptidoglycan only (20, 22).

The reason for muropeptide modification with NAP at the 1-carboxyl group of glutamic acid is still unknown. Cadaverine is necessary for the normal growth of *S. ruminantium* and has been implicated in the attachment of the outer membrane to the peptidoglycan layer, compensating for the missing lipoprotein (27). In this function, it could be replaced by putrescine or diaminohexane (23). These polyamines might directly connect the peptidoglycan layer to the outer membrane by interaction between their positively charged free amino groups and membrane phospholipids. In cyanelle peptidoglycan, this amino group is acetylated and direct interaction between peptidoglycan-bound NAP and the outer membrane surrounding the cyanelle seems unlikely. The NAP residues in cyanelle peptidoglycan might have some regulatory function for peptidoglycan synthesis and metabolism, or more likely they might alter the charge and polarity of the peptidoglycan layer. Both substitution and the reduced thickness compared with that of cyanobacterial peptidoglycan could thus increase the pervasiveness of the peptidoglycan network. This might be especially important for a cell organelle because of its need for extensive protein import from the cytoplasm.

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