N-ACETYLGLUCOSAMINYL-*N*-ACETYLMURAMYL-DIPEPTIDE, A NOVEL MUREIN BUILDING BLOCK FORMED DURING THE CELL DIVISION CYCLE OF *PROTEUS MIRABILIS*

Jobst GMEINER and Hein-Peter KROLL+

Institut für Mikrobiologie, Fachbereich Biologie Technische Hochschule Darmstadt, 6100 Darmstadt and +*Institut für Chemotherapie, Pharma-Forschungszentrum Bayer AG, 5600 Wuppertal 1, FRG*

Received 10 April 1981

1. Introduction

The murein (peptidoglycan) of Gram-negative bacteria consists of glycan chains containing alternating *N*-acetylated residues of glucosamine and muramic acid. The muramic acid residues are substituted by tetrapeptide units consisting of L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, part of which form crosslinkages between neighbouring glycan chains [1]. *Proteus mirabilis* murein is unique in regard to the *O*-acetylation of a portion of its *N*-acetylmuramic acid residues [2,3].

Working with synchronized cells of this organism and labelling the murein with radioactive *N*-acetyl-D-[1-^{14}C]glucosamine in vivo, we detected 2 new uncrosslinked building blocks after enzymatic digestion of the isolated murein with endo-*N,O*-diacetylmuramidase from *Chalaropsis*. Here, we present analytical data which identify these new compounds as *O*-acetylated and non-*O*-acetylated *N*-acetylglucosaminyl-*N*-acetylmuramyl-dipeptides.

2. Experimental

The methodology of the experiments leading to the detection of the new building blocks has been detailed in [4]. Briefly, cells of *Proteus mirabilis* wild-type strain 19 were grown in complex diluted medium, synchronized by sucrose density centrifugation and pulse labelled with *N*-acetyl-D-[1-^{14}C]-glucosamine. The radioactivity was then chased for several periods of time, cells were harvested, mixed with unlabelled cells and the polymeric murein sacculi were isolated. After digestion with endo-*N,O*-diacetylmuramidase from *Chalaropsis* the fragments were separated by gel filtration, desalted and chromatographed on silica gel thin-layer plates. The radioactive spots were eluted and 5–25 nmol of the compounds were analyzed by amino acid analysis after acid hydrolysis. The analyses were carried out using an LC 2000 automatic analyzer (Biotronic, Munich) directly connected to a Redi-Rac fraction collector (LKB, Bromma) via a magnetic valve controlled by the analyzer. Effluents containing glucosamine were collected in two 4 ml portions in szintivials, mixed with 16 ml Szintigel (Roth, Karlsruhe) and the radioactivity was determined in a Packard Tricarb liquid szintillation counter.

3. Results

3.1. *Appearance of novel murein building blocks during the cell cycle*

Under the experimental conditions employed synchronized cells of *Proteus mirabilis* exhibited an interdivision time of 55 min. At 35 min of the first cell cycle, a synchronized culture was pulse-labelled with radioactive *N*-acetyl-D-[1-^{14}C]glucosamine for 10 min. Subsequently, the radioactive *N*-acetylglucosamine was diluted 1:100, the culture was rapidly chilled and centrifuged. The sedimented cells were resuspended in medium containing unlabelled *N*-acetylglucosamine and incubated further. The cells started dividing ~10 min after resuspension and portions were removed for murein isolation at various time intervalls [4]. At 40 min of the chase period,

i.e., about one generation after the pulse, novel murein building blocks with higher R_F-values appeared as detected on thin-layer chromatograms of the monomer fraction (compare fig.1). The radioactivity in these compounds amounted to 4.8% of the radioactivity recovered with all spots, and increased with ongoing chase to 16.5% after 120 min.

The same experiment was then repeated cultivating synchronized cells in the presence of 6 μg penicillin G/ml which completely inhibited cell division, resulting in filamentous growth. Calculation of the amount of murein synthesized during the pulse period by the same number of cells according to the specific radioactivity in the glucosamine residues [4] showed a decrease of 48% in the presence of the antibiotic, as measured from cell samples removed immediately after resuspension for the chase. Despite this inhibition of overall murein synthesis due to the inhibition of septum formation, the novel murein building blocks appeared exactly at the same time. Amounts as high as 7.9% of the radioactive monomers were measured after 40 min chase, increasing to 20.7% after 120 min chase.

These results indicate that the new compounds isolated from insoluble polymeric murein were recovered mainly from murein regions synthesized during longitudinal extension of the murein sacculus.

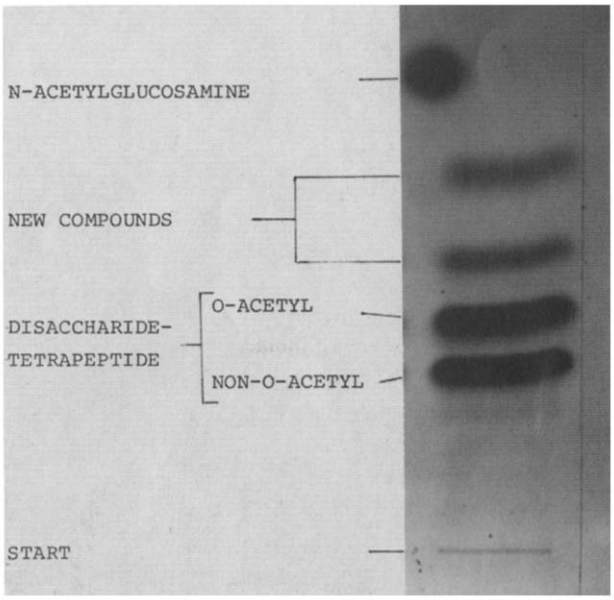

Fig.1. Thin-layer chromatography of the monomer fraction isolated from pulse-labelled murein, chased for 80 min in vivo in synchronized cells of *Proteus mirabilis*. The desalted monomer solution was applied to a silica gel thin-layer plate (Macherey and Nagel, Düren). The plate was developed twice in isobutyric acid/1 M ammonia (5:3, v/v). The bands were visualized by autoradiography. The migration distances relative to *N*-acetylglucosamine are listed in table 1.

3.2. *Isolation and analysis of the novel compounds*

The murein monomer fraction of the cell sample taken after 80 min chase was preparatively separated by thin-layer chromatography (fig.1). The 4 different bands were scraped off the plate, eluted with water and analyzed. Whereas, the fastest migrating band had the composition of a disaccharide-dipeptide, the second band was contaminated by free tetrapeptide which is inevitably produced in small amounts from monomers by limited β-elimination during handling and storage. This band, therefore, was further purified by high-voltage paper electrophoresis at 9000 V for 2 h. The radioactivity was detected as a sharp band 40.5 cm from the start, and it was analyzed again after elution from paper. The analytical data of all bands are given in table 1.

Both new compounds (disaccharide–dipeptide, *O*-acetylated; disaccharide–dipeptide, non-*O*-acetylated) showed an identical composition and differed from the known monomeric murein building blocks by lacking *meso*-diaminopimelic acid as well as one alanine residue. Since they itself differed only by their R_F-value as the 2 established monomer species do, we can safely conclude that the faster migrating compound represents the species with *O*-acetylated muramic acid. Samples of all bands were reduced with sodium borohydride. After acid hydrolysis and amino acid analysis, muramic acid could no longer be detected, confirming the disaccharide nature of the compounds not blocked at the reducing end.

From the percent distribution of glucosamine recovered with the 4 bands, it became evident that the novel building blocks comprised 4.6% of the total monomer fraction. Since the pulse-labelled cells were mixed with 100-times the number of unlabelled cells from a batch culture before murein isolation [4], and since ~50% of the total murein units are found as monomers [3,5] we can calculate that the murein sacculus of *Proteus mirabilis* contains 2–3% of such disaccharide–dipeptide units. The higher specific activity of these compounds (table 1, column 9), on the other hand, indicated that the murein synthesized during the pulse period was especially qualified for this kind of building block modification.

Table 1
Characterization of murein building blocks rcovered from the monomer fraction after thin layer chromatography

Compound	M_{GlcNAc}[a]	% of total GlcN recovered	Molar ratio[b]					Specific radioactivity of GlcN[c]
			Glu	Ala	A_2pm[c]	GlcN[c]	MurN[c]	
Disaccharide-dipeptide *O*-acetylated	0.80	2.0	1.00	0.98	0.08	0.91	0.85	34.7
Disaccharide-dipeptide non-*O*-acetylated	0.61	2.6	1.00	1.05	–	0.85	0.83	28.3
Disaccharide-tetrapeptide *O*-acetylated	0.50	41.2	1.00	1.89	0.99	0.95	0.78	12.5
Disaccharide-tetrapeptide non-*O*-acetylated	0.38	54.2	1.00	1.88	1.10	1.07	0.83	9.1

[a] Migration relative to *N*-acetylglucosamine on thin layer plates (see fig.1)
[b] Molar ratio relative to glutamic acid
[c] *Abbreviations:* A_2pm, meso-diaminopimelic acid; GlcN, glucosamine; MurN, muramic acid

4. Discussion

Detection of the desaccharide–dispeptides described was made possible in *Proteus mirabilis* by labelling murein specifically in the amino-sugar portion, since the unlabelled compounds cannot be detected by the conventional method of ninhydrin staining due to the lack of *meso*-diaminopimelic acid providing the free amino group. Likewise, such a disaccharide–dipeptide escapes detection in murein labelled in the *meso*-diaminopimelic acid residues as frequently performed using auxotrophs of *Escherichia coli.* It has been proven however that *E. coli* murein also contains such a compound (unpublished).

Our results of the pulse chase experiments with *Proteus mirabilis* clearly demonstrated that the novel building blocks were the result of murein modification after incorporation of normal building blocks into the murein polymer. Mainly murein of the cylindrical portion of the sacculus seemed to be susceptible to this type of modification at a specific time during the cell cycle, brought about by an as yet unknown enzyme insensitive to penicillin. Whether only monomers, or dimers as well, are substrates for the enzyme is not yet known.

What might be the advantage of such a modification? The escape of detection of these compounds by conventional methods suggests a possible answer: Only removal of *meso*-diaminopimelic acid residues from the peptide sidechains in a presumably well-defined murein region rigorously excludes any acceptor function of the murein glycan strands involved in attachment of new murein units via transpeptidationm since the ϵ-aminogroup of *meso*-diaminopimelic acid is the essential acceptor group for the transpeptidation reaction. Thus, the appearance of the novel building blocks might point to a new regulatory mechanism of murein biosynthesis during the division cycle. The fact that murein in the cylindrical part of the cell becomes modified only after a considerable time delay supports the conclusion that the modification process is involved in controlling initiation of new murein growing zones for cell elongation, perhaps regulating the direction of murein extension.

Acknowledgement

This investigation was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

[1] Weidel, W. and Pelzer, H. (1964) Adv. Enzymol. 26, 193–232.
[2] Fleck, J., Mock, M., Minck, R. and Ghuysen, J. M. (1971) Biochim. Biophys. Acta 233, 489–503.
[3] Martin, H. H. and Gmeiner, J. (1979) Eur. J. Biochem. 95, 487–495.
[4] Gmeiner, J. and Kroll, H.-P. (1981) submitted.
[5] Gmeiner, J. (1979) Arch. Mikrobiol. 121, 177–180.