Characterization of a β-N-acetylmuraminidase of *Escherichia coli* and Elucidation of Its Role in Muropeptide Recycling and β-Lactamase Induction*

Received for publication, June 2, 2000, and in revised form, August 16, 2000
Published, JBC Papers in Press, September 7, 2000, DOI 10.1074/jbc.M004797200

Walter Vötsch and Markus F. Templin‡

From the Max-Planck-Institut für Entwicklungsbiologie, Abteilung Biochemie, Speemannstrasse 35, 72076 Tübingen, Germany

Using the known mapping position the gene encoding a β-1,4-N-acetylmuraminidase needed for the degradation of muropeptides could be identified. nagZ encodes a cytosolic enzyme active on N-acetylmuramyl-β-1,4-(1,6)-anhydromuramic acid containing muropeptides. These degradation products of the peptidoglycan are formed during the enlargement of the murein sacculus as a consequence of a growth mechanism, which couples the controlled degradation of the cell wall polymer with the insertion of new material. NagZ is needed for the formation of monosaccharides from the released disaccharides during the cytosolic steps of the muropeptide-recycling pathway. The formation of intracellular 1,6-anhydro-N-acetylmuramylmuramidopeptidase is important for the expression control of the inducible β-lactamases of the AmpC type. A mutant lacking active NagZ cannot establish AmpC mediated β-lactam resistance. The biochemical characterization of the enzyme showed its activity on different muropeptides and inhibitors of enzyme activity could be identified. This observation might be important for designing inhibitors of NagZ that could prevent the establishment of β-lactam resistance of *Enterobacteria* possessing inducible β-lactamases.

Bacteria are enclosed by an exoskeletonal structure found in the cell envelope (1). In most cases the bag-shaped macromolecule murein (peptidoglycan) stabilizes the cell mechanically and determines the shape of the bacterium (2). It is a heteropolymer, made up of glycan strands composed of an alternating sequence of two amino-sugars (N-acetyluramuramic acid and N-acetylglycosamine) linked by β-1,4 glycosidic bonds (reviewed by Höltje in Ref. 3). The glycanes are interlinked by short peptide bridges, and a covalently closed network completely surrounding the cell is formed. To enlarge this structure not only does new material have to be synthesized, it has to be integrated into the existing murein. This is accomplished by the concerted action of murein synthases and murein hydrolyases (3–6). During the controlled enlargement of the exoskeleton, old material from the sacculus gets released as turnover products while the polymer grows by the insertion of new murein glycan strands (7–9). Different enzymatic activities have been
described to take part in this turning over of the murein sacculus. Lytic transglycosylases are muramidases that are degrading the glycan strands to anhydro-disaccharides (10), endopeptidases cleave peptide cross-links (11–13) and N-acetylmuramyl-L-alanine amidases release oligopeptides from the murein or from muropeptides (7). As a result of the action of these enzymes, disaccharides (N-acetylmuramyl-β-1,4-(1,6)-anhydro-N-acetylmuramic acid) are formed that carry a characteristic 1,6-anhydro ring structure at the muramic acid.

For *Escherichia coli* the turnover process has been well studied, and it could be shown that during one generation up to 50% of the cell wall gets degraded as a result of normal growth processes (9, 14). These breakdown products are efficiently reutilized by *E. coli* in a recycling process; it could be shown that this reuse is an important source for the formation of new murein precursors (15). A major metabolic pathway, describing the fate of the peptide moiety originating from the breakdown of the murein could be elucidated. During the early steps of the recycling, the periplasmic degradation products get taken up by membrane transporters. Oligopeptides are internalized by the MmpA/Opp system (16, 17), whereas the membrane permease AmpG transports sugar containing muropeptides (18).

In the cytosol the muropeptides get further degraded by the action of an N-acetylmuramyl-L-alanine amidase (AmpD; Refs. 19 and 20) and an essential LD-carboxypeptidase (LdcA; Ref. 21) and l-alanyl-l-glutamyl-meso-diaminopimelic acid gets released. The tripeptide is recognized by the muropeptide ligase Mpl (22) and added to UDP-N-acetylmuramic acid (UDP-MurNAc),1 and UDP-MurNAc-tripeptide, a precursor for murein synthesis, is formed.

The fate of the sugar moiety is not clear despite the fact that the presence of a β-1,4-N-acetylmuraminidase active on muropeptides had been reported over 20 years ago (23, 24). Here the identification of the gene encoding NagZ, a cytosolic glucosaminidase involved in muropeptide recycling, is shown. Additionally, its need for the expression control of certain β-lactamases is described.

EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Growth Conditions**

The β-1,4-N-acetylmuraminidase-deficient mutant E593 (24) is derived from *E. coli* K-12 AB1157 (F Δgpt-proA62) argE3 his-4 leu-6 thr-1 ara-14 galK2 lacY1 xyl-5 mtl-1 thi-1 supE44 rpsL31 Str+ (λr3). The general cloning host was XL-1 blue (Stratagene, La Jolla, CA). To examine β-lactamase induction, strains were transformed with

* This work was supported by European Commission Project BIOU-CT96-0122. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Present address: NMI, an der Universität Tübingen, Markwiesenstr. 55, 72770 Reutlingen, Germany. Tel.: 49-7121-51530-802; Fax: 49-7121-51530-16; E-mail: Templin@nmi.de.

1 The abbreviations used are: MurNAc, N-acetylmuramic acid; anhydro-disaccharide, GlcNAc-β-1,4-(1,6)-anhydro-MurNAc; PNP, p-nitrophenol; rpHPLC, reversed phase high pressure liquid chromatography; PCR, polymerase chain reaction.
pPJ1 (9), a plasmid containing the amprR-ampC operon from Enterobacter cloaceae (25). pBC SK' (Stratagene, La Jolla, CA) was used as cloning vector and pQE30 (Qiagen, Hilden, Germany) for construction of a Hiss-tagged NagZ protein. Bacteria were cultivated aerobically at 37 °C in LB medium. Growth was monitored by determination of A578 by addition of 0.5 mM isopropyl-D-galactoside (IPTG).

Agar Diffusion Test

LB agar plates (1.5% agar) were overlaid with 3 ml of LB top-agar (0.7% agar) containing 10 µg/ml kanamycin, 12.5 µg/ml chloramphenicol, or 50 µg/ml ampicillin in LB broth or on LB agar plates.

Purification of Muropeptides

Murein sacculi were isolated as described before and treated with α-amylase and pronase (30). Murein from 2 liters of culture was completely digested into noncross-linked disaccharide units by incubation with purified MepA (3 µg) and purified Shi70 (2 µg) in a volume of 4 ml of buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.0). Anhydro-disaccharide-tripeptide and anhydro-disaccharide-tetrapeptide were isolated by rpHPLC (30). To obtain unsubstituted anhydro-disaccharides isolated sacculi were degraded with purified Shi70 (2 µg) and isolated AmIC (4 µg). The anhydro-disaccharides were isolated by rpHPLC on 5-µm Hypersil-ODS (4.6 mm x 125 mm; Bischoff, Leonberg, Germany). Chromatography was performed at room temperature at a flow rate of 1 ml/min using 0.1% trifluoroacetic acid. After 10 min a gradient (0% to 30% acetonitril) that was built up in 10 min was applied before equilibrating to 0.1% trifluoroacetic acid for 10 min. Fractions containing muropeptides were collected and dried.

DNA Manipulations and PCR

Standard techniques were used for manipulating the plasmid DNA (26). E. coli was transformed as described by Inoue et al. (27). Restriction endonucleases were purchased from Roche Molecular Biochemicals; oligonucleotides came from MWG-Biotech (Ebersberg, Germany). PCR (28) was performed using PowerScript polymerase (PAN Systems, Nu¨rnberg, Germany) in a volume of 50 µl containing 10 mM Tris, 50 µM dNTPs, 0.4 -AAAGCTGCTATTAAGCTT-3' and 5'-AAACGTCGACGCTTCCACATAAAGGC-3') and cloned into the EcoRI and SnaI sites of the vector pBC SK' (Stratagene, La Jolla, CA). The constructs were termed pBC-nagZ and pBC-nagZmut, respectively. The addition of an N-terminal His6 tag to the target protein was obtained after cloning of the PCR product (template chromosomal DNA from E. coli AB1157; primers: 5'-GAATTCCTGGTCTAAGTGGG-3' and 5'-AAGATCATGCGTCCCTGCGTCCGAACTCCATAGGC-3') cloned into the EcoRI and SnaI sites of the vector pBC SK' (Stratagene, La Jolla, CA). The constructs were termed pBC-nagZ and pBC-nagZmut, respectively. The addition of an N-terminal His6 tag to the target protein was obtained after cloning of the PCR product (template chromosomal DNA from E. coli AB1157; primers: 5'-AAACGTCGACGCTTCCACATAAAGGC-3') into pQE30 (Qiagen) cut with BamHI and SnaI. The resulting plasmid was named pHN5. All constructs were sequenced; in the case of the mutant the entire sequence was independently obtained clones were subject to sequencing.

Cloning of nagZ and Construction of an Overproducing System

The coding sequence of yeast was amplified by using PCR. Both the 5' (from E. coli AB1157) and the copy from the glucosaminidase-deficient route (E. coli E593) were used as templates (specific primers 5'-AAACGTCGACGCTTCCACATAAAGGC-3') and cloned into the EcoRI and SnaI sites of the vector pBC SK' (Stratagene, La Jolla, CA). The constructs were termed pBC-nagZ and pBC-nagZmut, respectively. The addition of an N-terminal His6 tag to the target protein was obtained after cloning of the PCR product (template chromosomal DNA from E. coli AB1157; primers: 5'-AAACGTCGACGCTTCCACATAAAGGC-3') into pQE30 (Qiagen) cut with BamHI and SnaI. The resulting plasmid was named pHN5. All constructs were sequenced; in the case of the mutant the entire sequence was independently obtained clones were subject to sequencing.

Purification of NagZ

To overproduce the His6-tagged NagZ 800 ml of culture of E. coli XL-1 blue harboring pHN5 were grown in LB medium with moderate shaking at 37 °C. Expression of the protein was induced at an A578 of 0.3 by addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) after 4 h. The cells were harvested by centrifugation, washed with 50 ml ice-cold buffer A (100 mM sodium phosphate, 100 mM NaCl, pH 7.0), and resuspended in 5 ml buffer A containing 10 µM imidazol (buffer B). After disrupting the cells by three passages through a precooled French presser at 15,000 p.s.i. and centrifugation (50 min, 20,000 x g), the supernatant was used for purification of the enzyme by metal chelation chromatography on Superflow nickel-nitritoracetate (Qiagen). After loading of the column (bed volume, 3 ml; equilibrated with buffer B), the column was washed with 60 ml of buffer B at a flow-rate of 4 ml/h. The protein was eluted with buffer A containing 250 mM imidazol. Glucosaminidase activity in the eluted fractions was determined by measuring hydrolysis of PNP-β-GlcNac, and fractions showing high activity were pooled. After extensive dialysis against buffer A, the protein was stored at −20 °C.

RESULTS

Identification and Molecular Cloning of the nagZ Gene—

Mapping data from Hrebenda (33) and Park (15) suggest a chromosomal location for the gene of a β-1,4-N-acetylglucosaminidase active on muropeptides at around 26 min of the E. coli genome. To identify this gene, the possible reading frames from this region (25 min to 27 min) were subject to database comparisons using the BLAST algorithm (34) on the Swiss Prot data base. One of the approximately 100 screened coding frames showed a significant similarity to known glycosyl hydrolases. To show that such an enzyme is encoded by yeast (located at 25.1 min, 1163 kilobase pairs of the physical map of the E. coli genome), the gene was amplified by PCR and subcloned into the cloning vector pBC SK' . Transformation of E. coli E593, a mutant deficient in β-1,4-N-acetylglucosaminidase activity, with this plasmid restored enzyme ac-
tivity as detected with a whole cell β-N-acetylglucosaminidase assay. Transformation of the same strain with a plasmid harboring a plasmid with the ycfO allele from E593 showed no measurable increase in glucosaminidase activity (data not shown). Both alleles were sequenced, and it could be shown that the wt copy is identical to the published sequence of ycfO (35). For the mutant allele a single base pair change was detected (changing Gly101 to Asp) and therefore is responsible for the total loss of activity. The identified open reading frame was renamed to nagZ as proposed by Park (15).

Sequence Analysis—The reading frame from the ycfO gene encodes a protein of a predicted size of 37 kDa. Using the TopPredII algorithm (36), no leader sequence was detected; therefore, a cytosolic localization is expected. These results are in agreement with data from Yem and Wu (23), who characterized the cytosolic β-N-acetylglucosaminidase from E. coli AB1157 mapping at 25 min.

Further similarity searches (using the PSI BLAST algorithm; Ref. 37) performed with the coding sequence of NagZ put the protein to family III of glycosyl hydrolases, a group of enzymes mainly containing β-glucosidases. Recently, the active site aspartic acid of ExoII, a highly similar protein from Vibrio furnissi was identified (38, 39), and, indeed, this amino acid is conserved in all members of the family including the newly characterized enzyme. The mutation found in the nagZ allele from E593 maps close to another conserved region in the protein (data not shown). Highly similar orthologs were found within most Enterobacteriaceae sequenced to date, and searching the unfinished microbial genomes data base (last update, May 16, 2000 at the National Center for Biotechnology Information) revealed the presence of further homologues (in Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Yersinia pestis, Pasteurella multocida, Vibrio cholerae, Hemophilus influenzae, Hemeophilus ducreyi, Klebsiella pneumonia, Pseudomonas aeruginosa, Shewanella putrefaciens, Pseudomonas putida, Salmonella enteritidis, Neisseria meningitidis, Bordetella pertussis, Neisseria gonorrhoeae, and Bordetella bronchiseptica; e values in a tblastn gapped blast search < e –61). This suggests a conserved function for NagZ.

Characterization of a nagZ Mutant—Initially, no obvious phenotype for E593, the mutant isolated by Yem and Wu (23, 24) was observed. Neither growth rate or cell shape was changed in LB medium (data not shown). To find out whether NagZ is indeed an enzyme that is involved in muropeptide recycling, an indirect approach was chosen. Muropeptides are known effectors of the transcriptional activator AmpR that controls the expression of AmpC type β-lactamases. This activator promotes β-lactamase transcription and is needed to establish β-lactam resistance. Activation of AmpR only occurs upon binding of certain muropeptides that are formed during the cytosolic degradation steps of turnover material from the cell wall. During normal growth conditions, the intracellular concentration of these muropein metabolites stays low because they are efficiently recycled. In bacteria treated with β-lactams (e.g. cefoxitin), a high amount of muropeptides gets released and accumulates in the cytoplasm. Consequently, AmpR activation is found, and AmpC production is induced (18). The muropeptides activating AmpR are anhydro-monoaccharides (anhydro-N-acetyl-muramoyl-peptides) that are products of an N-acetyl-glucosaminidase acting on the released turnover material. Therefore, a strain carrying a nagZ mutation was tested for its capability to induce expression of AmpC type β-lactamasas by determination of the sensitivity against β-lactam antibotics. E. coli E593 was transformed with pJP1 (9), a plasmid that carries the ampR-ampC operon from E. cloacae, and the influence of the nagZ mutation on the induction process was examined. In an agar diffusion assay the sensitivity to cefoxitin, a cephalosporin that is a potent inducer of AmpC expression, was determined. In the corresponding control strain (AB1157), the transformation with pJP1 leads to high resistance against β-lactams. In contrast, the mutant E593 harboring the plasmid stayed as sensitive against cefoxitin as a strain not possessing the resistance plasmid (Table I). Introducing a second plasmid containing the cloned wild type allele of nagZ can complement this defect; the control transformation with the mutated nagZ from E593 on a plasmid did not lead to increased resistance to the cephalosporin. Therefore, it is concluded that the identified glucosaminidase is involved in muropeptide recycling and is needed for the establishment of expression of the inducible β-lactamases of the AmpC type.

To get further insight into the induction process, β-lactamase induction was measured in cultures of AB1157 and E593 carrying pJP1 (ampR ampC) after treatment with 2.5 µg/ml cefoxitin. Activity was determined in culture aliquots (taken every 15 min) in a newly established whole cell assay (see “Experimental Procedures”). The hydrolysis of nitrocefin, a chromogenic substrate for β-lactamases was monitored, and induction factors could be calculated for the strains carrying the resistance plasmid (Table II). For the control a 17-fold induction (as compared with the untreated control) was found after 45 min, in the mutant the calculated induction factor was 4.5. Still a substantial amount of β-lactamase (37.2 nano mol/min × A578) was measured in the induced nagZ mutant. Al-

### Table I

Sensitivity of a nagZ mutant against cefoxitin

| Strain                      | Zone of Inhibition (Diameter) | MIC µg/ml |
|----------------------------|-------------------------------|-----------|
| AB1157 (wild type)         | 36                            | 1         |
| AB1157, pJP1               | 23                            | 8         |
| AB1157, pJP1, pBC SK       | 23                            | 8         |
| AB1157, pJP, pBC-NagZ      | 25                            | 4         |
| AB1157, pJP, pBC-NagZmut   | 24                            | 8         |
| E593 (nagZ)                | 38                            | 1         |
| E593, pJP                  | 38                            | 1         |
| E593, pJP1, pBC SK         | 37                            | 1         |
| E593, pJP1, pBC-NagZ       | 25                            | 4         |
| E593, pJP1, pBC-NagZmut    | 38                            | 1         |

* A nagZ mutant and its corresponding parent were transformed with a plasmid carrying the ampR ampC operon from E. cloacae and with different derivatives of the cloning vector pBC SK.† containing wt nagZ or mutated nagZmut.

† Sensitivity was determined in an agar diffusion assay. Filter disks (5 mm in diameter) loaded with 200 µg of cefoxitin were placed onto an agar plate inoculated with the indicated strain. After incubation overnight the diameter of the zone of inhibition was measured.

‡ The minimal inhibitory concentration (MIC) was determined on agar plates containing the indicated concentration of the antibiotic.

### Table II

β-Lactamase induction in a nagZ mutant carrying ampC/ampR from E. cloacae

| Strain | Not induced | Induced |
|--------|-------------|---------|
| AB1157, pJP1 | 4.6 4.6 (1.0) | 5.1 5.2 (1.0) |
| 15 min  | 5.4 9.3 (1.7) | 5.3 8.0 (1.5) |
| 30 min  | 6.5 50.6 (7.8) | 7.7 23.9 (3.1) |
| 45 min  | 6.2 106.9 (17.1) | 8.3 37.2 (4.5) |

The reading frame from the ycfO gene encodes a protein of a predicted size of 37 kDa. Using the TopPredII algorithm (36), no leader sequence was detected; therefore, a cytosolic localization is expected. These results are in agreement with data from Yem and Wu (23), who characterized the cytosolic β-N-acetylglucosaminidase from E. coli AB1157 mapping at 25 min. Further similarity searches (using the PSI BLAST algorithm; Ref. 37) performed with the coding sequence of NagZ put the protein to family III of glycosyl hydrolases, a group of enzymes mainly containing β-glucosidases. Recently, the active site aspartic acid of ExoII, a highly similar protein from Vibrio furnissi was identified (38, 39), and, indeed, this amino acid is conserved in all members of the family including the newly characterized enzyme. The mutation found in the nagZ allele from E593 maps close to another conserved region in the protein (data not shown). Highly similar orthologs were found within most Enterobacteriaceae sequenced to date, and searching the unfinished microbial genomes data base (last update, May 16, 2000 at the National Center for Biotechnology Information) revealed the presence of further homologues (in Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Yersinia pestis, Pasteurella multocida, Vibrio cholerae, Hemophilus influenzae, Hemophilus ducreyi, Klebsiella pneumonia, Pseudomonas aeruginosa, Shewanella putrefaciens, Pseudomonas putida, Salmonella enteritidis, Neisseria meningitidis, Bordetella pertussis, Neisseria gonorrhoeae, and Bordetella bronchiseptica; e values in a tblastn gapped blast search < e –61). This suggests a conserved function for NagZ.

Characterization of a nagZ Mutant—Initially, no obvious phenotype for E593, the mutant isolated by Yem and Wu (23, 24) was observed. Neither growth rate or cell shape was changed in LB medium (data not shown). To find out whether NagZ is indeed an enzyme that is involved in muropeptide recycling, an indirect approach was chosen. Muropeptides are known effectors of the transcriptional activator AmpR that controls the expression of AmpC type β-lactamases. This activator promotes β-lactamase transcription and is needed to establish β-lactam resistance. Activation of AmpR only occurs upon binding of certain muropeptides that are formed during the cytosolic degradation steps of turnover material from the cell wall. During normal growth conditions, the intracellular concentration of these murein metabolites stays low because they are efficiently recycled. In bacteria treated with β-lactams (e.g. cefoxitin), a high amount of muropeptides gets released and accumulates in the cytoplasm. Consequently, AmpR activation is found, and AmpC production is induced (18). The muropeptides activating AmpR are anhydro-monoaccharides (anhydro-N-acetyl-muramoyl-peptides) that are products of an N-acetyl-glucosaminidase acting on the released turnover material. Therefore, a strain carrying a nagZ mutation was tested for its capability to induce expression of AmpC type β-lactama-
though this level of β-lactamase production does not lead to any increase in β-lactam resistance, it opens the question how induction can occur in the nagZ mutant. A possible explanation might be the presence of a second enzyme able to hydrolyze anhydro-muropeptides. This enzyme could be identical to BglX, a periplasmic β-D-glucosidase also belonging to the family III of the glycosyl hydrolases (40). This enzyme, together with an transporter specific for the released anhydro-monosaccharides would constitute an unknown way to reutilize turnover material.

Purification of His$_6$-NagZ—To allow a simple purification of the identified enzyme, the coding region of nagZ was subcloned into the gene fusion vector pQE30 (Qiagen), and an N-terminal His$_6$ extension was created. The fusion protein was active in the whole cell glucosaminidase assay (Ref. 23 and data not shown). A high degree of overexpression of the soluble fusion protein could be obtained upon addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (Fig. 1). Metal chelation chromatography was used for purification on a nickel-nitrilotriacetate-Sepharose column, and a simple one-step purification procedure was established. Essentially pure protein as judged by SDS-polyacrylamide gel electrophoresis and Coomassie staining could be obtained (Fig. 1). The apparent molecular mass (37 kDa) agrees with the calculated value from the predicted amino acid sequence. The protein concentration was found to be 0.3 mg/ml, and the specific activity on PNP-β-1,4-N-acetylglucosaminidase and β-Lactamase Induction 39035

Characterization of NagZ—An initial characterization of the enzyme was performed using PNP-β-GlcNAc, a chromogenic substrate for β-1,4-glucosaminidases that had been shown to be accepted by NagZ (23). In a buffer system containing 50 mM Hepes, the pH optimum of the enzyme was detected at 7.1, and the temperature optimum was found at 38 °C. The addition of 1 M NaCl stimulated the activity 5-fold (Fig. 2). All experiments described below were therefore performed in 50 mM Hepes buffer, pH 7.1, containing 0.5 mM NaCl. The apparent kinetic constants with PNP-β-GlcNAc as substrate (calculated from a Lineweaver-Burk diagram) were: $K_m$ 310 μM, $V_{max}$ 13.3 μmol/min mg protein at 25 °C.

Activity of NagZ on Muropeptides—The role of NagZ in muropeptide recycling suggests three possible natural substrates. The major muropeptide released during cell wall turnover by E. coli is the anhydro-disaccharide-tetrapeptide (41). This disaccharide can be converted to anhydro-disaccharide and to anhydro-disaccharide-tripeptide by the action of the intracellular amidase AmpD (19) and the LD-carboxypeptidase LdcA (21), respectively. To test activity of NagZ on these muropeptides, they were isolated in a larger scale. Isolated murein sacculi were degraded to anhydro-disaccharides by incubation with isolated Slt70 (42); cross-linked muropeptides were converted to anhydro-disaccharide-tetrapeptide and anhydro-disaccharide-tripeptide by enzymatic cleavage of the cross-bridge by addition of purified MepA (11). Disaccharides lacking the peptide side chain were obtained from isolated sacculi treated with purified AmiC2 and isolated Slt70. The muropeptides were isolated by rpHPLC and quantified using authentic standards (kindly provided by A. Ursinus). An initial characterization showed that the isolated NagZ accepts all three muropeptides as substrates (data not shown).

Kinetic Characterization and Inhibition of NagZ—To find

2 M. Templin, manuscript in preparation.
out about a preference for any of these substrates, a kinetic characterization of the action of NagZ on the three muropeptides was performed. The substrate concentration was varied from 1 to 300 μM, and the initial reaction velocity was estimated using the rpHPLC based assay (Fig. 3A). The apparent kinetic constants for the different muropeptides were calculated with the EKI data analysis program that uses a nonlinear regression of the Michaelis-Menten equation (43). The \(K_m\) values determined were 32 μM for anhydro-disaccharide-tetrapeptide, 29 μM for anhydro-disaccharide-tripeptide, and 35 μM for anhydro-disaccharide. These values are nearly identical; it is concluded that all muropeptides formed during the turnover and recycling process are substrates for NagZ.

In the assay employed, strong inhibition of enzyme activity was observed at high substrate concentrations. The graphical presentation of the kinetic data in a Lineweaver-Burk diagram visualizes this effect (Fig. 3B). The apparent inhibition constant for the anhydro-disaccharide-tetrapeptide was estimated from a Dixon plot and found to be approximately 100 μM. Although this value is higher then the amount of muropeptides found during normal growth conditions, it is in the range of the intracellular muropeptide concentration found during \(\beta\)-lactamase induction.

The observation that NagZ activity can be inhibited was further studied. The influence of different amino-sugars on enzyme activity was determined. Strong inhibition was found with N-acetylglucosamine, a product of the glucosaminidase and with the chromogenic substrate of the enzyme the PNP-β-N-acetylglucosamine-6-P. Bulgecin, MurNAc, GlcNAc-6-phosphate, and chitobiose had no effect on the enzyme. Interestingly, bulgecin, a glycopeptide that has been shown to inhibit the soluble lytic transglycosylase Slh70, did inhibit NagZ activity (Table III). In vivo, inhibitors of NagZ need to be present in the cytosol, and uptake systems internalizing such compounds are needed. Because the identified inhibitors are all different amino-sugars, it seems possible that known sugar transporters will facilitate the uptake of such compounds.

**DISCUSSION**

By reusing the turnover material, which is released during growth of the murein sacculus, *E. coli* saves a substantial amount of energy. Indeed, the recycling process has to be seen as a major energy saving pathway by which precursors for cell wall synthesis are formed. During exponential growth about 50% of the cell wall gets released per generation, and about a third of the peptides found in newly formed precursor molecules originate from recycled material (44). With the identification of the uptake systems (MppA/Opp and AmpG), the tripeptide releasing enzymes (AmpD and LdcA) and the muropeptide ligase Mpl all enzymes needed for the reuse of the peptide are known (15). In contrast, it is not clear what happens with the disaccharide moiety taken up by the cell. Although the presence of an intracellular \(\beta\)-1,4-N-acetylglucosaminidase active on muropeptides has been described (23), its physiological function has not been clarified. This work could show that this enzyme leads to the formation of cytosolic monosaccharides. For one of the reaction products of NagZ, N-acetylglucosamine, a reutilization by feeding it into the described metabolic pathways using this amino-sugar seems likely. Still, a cytosolic sugar kinase converting this amino-sugar into N-acetylglucosamine-phosphate has to be identified. The other product, 1,6-anhydro-muramic acid substituted with

---

**TABLE III**

**Inhibition of NagZ activity by different amino sugars**

| Concentration (mM) | Inhibition (%) |
|--------------------|---------------|
| N-Acetylglucosamine | 0.2           | 70  |
|                    | 1             | 92  |
|                    | 5             | 98  |
| N-Acetylmuramic acid | 5             | <2  |
| N-Acetylglucosamine-6-P | 5          | <5  |
| Bulgecin           | 0.056         | <2  |
|                    | 0.28          | 8   |
|                    | 1.4           | 44  |
| Chitobiose         | 0.2           | 7   |
|                    | 1             | 17  |
|                    | 5             | 52  |
| PNP-β-N-acetylglucosamine | 0.2       | 45  |
|                    | 1             | 79  |
|                    | 5             | 95  |

*Inhibition of NagZ was determined in the rpHPLC based assay with 50 ng of purified His6-NagZ and 50 μM anhydro-disaccharide-tetrapeptide as substrate under standard conditions.
a side peptide at the lactyl moiety is subject to further enzymatic degradation. The action of the cytosolic N-acetylmuramyl-L-alanine amidase AmpD leads to the release of the anhydro-muramic acid. This is an unusual sugar containing an intramolecular ring from C-1 to C-6 formed during the enzymatic cleavage of the β-1,4-glycosidic bond between the N-acetylmuramic acid and the N-acetylglucosamine by the action of the lytic transglycosylases. These muramidases catalyze the cleavage of the glycosidic bond found in the glycan strands of the polymer during the turnover of the murein sacculus. The cleavage occurs not by hydrolysis but by an intramolecular transglycosylation reaction (10, 45). Part of the energy released during splitting of the bond gets used to create the intramolecular 1,6-ring, and anhydro-muropeptides are formed. It has been argued that this is a way of saving energy that can be used during the metabolism of the anhydro-sugars (46). Therefore the reuse of 1,6-anhydro-muramic acid might be accomplished by a way different from known sugar utilizing systems. A phosphorylation mechanism taking advantage of the energy stored in the intramolecular ring might be involved in metabolizing these sugars. Even a direct conversion into an UDP activated muramid acid, an early precursor for cell wall synthesis, might be envisioned.

The muropeptide recycling pathway is not only important for saving energy, it is used for signaling and sensing processes in Gram-negative bacteria (47, 48). During growth, turnover material is released in a controlled manner and gets recycled. Disturbance of this process, either by mutations in the recycling pathway or by interfering with cell wall metabolism, leads to changes in the pools of intracellular molecules formed during their reuse (48, 49). Gram-negative bacteria are able to detect deviations from normal conditions and thereby sense the state of the cell wall. Work on the inducible β-lactamases of the AmpC type has shown how pools of intracellular metabolites from degradation and synthesis of the murein are used for signaling. Changes in the amount of the anhydro-N-acetylmuramyl-peptides (tri-, tetra-, or pentapeptides) lead to differences in the expression pattern of the AmpC β-lactamases (41). The sensing device coupling the muropeptide recycling pathway to precursor synthesis is the transcriptional activator AmpR. It has the ability to complex UDP-MurNAc-pentapeptide and stays active upon binding. In the presence of high amounts of the anhydro-N-acetylmuramyl-peptides, this murein precursor gets displaced, and AmpR gets functional as transcriptional activator (48). Therefore, by measuring the relative amounts of precursors and degradation products, information about the state of the cell wall can be obtained. In the case of the inducible β-lactamase, the increased degradation of the murein caused by the presence of β-lactams is detected and leads to expression induction of AmpC. Actually most of the proteins needed for recycling have been identified during studies of the expression control of β-lactamases, and only later could their primary function be elucidated (15). Here an opposing approach was chosen. By identifying and characterizing an enzyme needed for muropeptide recycling, information about its role in the establishment of β-lactam resistance was obtained. The need of a β-1,4-N-acetylglicosaminidase for the formation of the positive effectors (anhydro-N-acetylmuramylpeptides) of AmpR is obvious because they are formed as reaction products of this enzymatic activity. Consequently, a mutant lacking NagZ is not able to produce the activating monosaccharides. This work could show that the enzyme is indeed needed for establishing the resistant state. The sensitivity against β-lactams that is found for a nagZ mutant transformed with a plasmid carrying the ampRC operon is comparable with a situation where no ampC gene is present. Therefore, the inactivation of this glucosaminidase can be seen as a possibility to inhibit expression of inducible β-lactamases and lock bacteria capable of producing AmpC in a sensitive state.

The biochemical characterization of the identified β-1,4-N-acetylglicosaminidase NagZ demonstrated some properties of the enzyme that are of interest in this context. The possibility to inhibit enzyme activity by addition of various amino-sugars might allow the design of specific inhibitors of NagZ. Such compounds would act as indirect effectors on AmpC expression and prevent expression induction of AmpC type β-lactamases. These enzymes are important virulence factors in a variety of pathogenic Gram-negative microorganisms, and novel approaches to treat infections caused by these bacteria are needed. By using inhibitors of NagZ, production of β-lactamases could be prevented, and strains resistant against β-lactam antibiotics could be rendered sensitive again.

Acknowledgment—I thank Joachim-Volker Hölte for support throughout these studies.

Note Added in Proof—During the review process of this paper the group of Ted Park published a paper describing the cloning of the nagZ gene: Cheng, Q., Li, H., Merdek, K., and Park, J. T. (2000) _J. Bacteriol_. 182, 4836–4840.
39038

β-1,4-N-acetylglucosaminidase and β-Lactamase Induction

38. Claros, M. G., and von Heijne, G. (1994) Comput. Appl. Biosci. 10, 685–686
39. Altschul, S. F., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 444–447
40. Vocadlo, D. J., Mayer, C., He, S., and Withers, S. G. (2000) Biochemistry 39, 117–126
41. Chitlaru, E., and Roseman, S. (1996) J. Biol. Chem. 271, 33433–33439
42. Yang, M., Luoh, S. M., Goddard, A., Reilly, D., Henzel, W., and Bass, S. (1996) Microbiology 142, 1659–1665
43. Wiedemann, B., Dietz, H., and Pleifle, D. (1998) Clin. Infect. Dis. 27, (Suppl. 1) 42–47
44. Templin, M. F., Edwards, D. H., and Hölte, J. V. (1992) J. Biol. Chem. 267, 20039–20043
45. Bisswanger, H. (1994) Enzymkinetik. Verlag Chemie, Weinheim, Germany
46. Park, J. T. (1995) Mol. Microbiol. 17, 421–426
47. Thunnissen, A. M., Isaacs, N. W., and Dijkstra, B. W. (1995) Proteins 22, 245–258
48. Hölte, J. V. (1995) Arch. Microbiol. 164, 243–254
49. Jacobs, C. (1997) Science 278, 1731–1732
50. Jacobs, C., Frere, J.-M., and Normark, S. (1997) Cell 88, 823–832
51. Kohlrausch, U., and Hölte, J. V. (1991) J. Bacterial. 173, 3425–3431