JOURNAL OF BACTERIOLOGY, July 1976, p. 319-326

Vol. 127, No. 1
Printed in U.S.A.

Amidase Activity Involved in Peptidoglycan Biosynthesis in Membranes of *Micrococcus luteus (sodonensis)*

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Received for publication 13 February 1976

Membrane suspensions prepared from *Micrococcus luteus (sodonensis)* in both the exponential and stationary phases of growth contained a transglycosidase activity capable of synthesizing linear peptidoglycan. Exponential-phase membranes also contained an *N*-acetylmuramyl-L-alanine amidase activity which degraded the peptidoglycan as it was formed. The product of this amidase was purified and found to be free pentapeptide. The amidase was specific for peptidoglycan and could not attack lower-molecular-weight substrates even though the susceptible bond was present. Crude cell wall preparations isolated from exponential-phase cells also contained high levels of amidase. This cell wall-bound amidase would preferentially degrade in vitro-synthesized peptidoglycan over its own cell wall. Amidase activity could be solubilized from both cell walls and membranes by Triton X-100 treatment, butanol extraction, or LiCl extraction. Both membrane- and cell wall-derived amidases, solubilized by LiCl extraction, appeared to be of high molecular weight (greater than 150,000). Once solubilized, these wall- and membrane-derived amidases could attack the cross-bridged peptidoglycan of purified native cell walls, whereas bound amidases could not.

Peptidoglycan biosynthesis has been studied in cell-free systems from *Micrococcus luteus* (*lysodeikticus*) where isolated membranes serve as a source of enzymes (1, 3). *M. luteus* (*sodonensis*) has a peptidoglycan structure with many of the same structural features as that of *M. luteus* (*lysodeikticus*) albeit a marked difference in lysozyme susceptibility (6, 11). *N*-acetylmuramyl-L-alanine amidase activity has been postulated to be part of the biosynthetic pathway of the peptidoglycan, since the pathway contains cross-bridging peptides made up of substituent peptides linked "head to tail" (6, 19). Amidase activity was not detected in membrane preparations from *M. luteus* (*lysodeikticus*), but Mirelman et al. (14, 15) did obtain indirect evidence of amidase activity in synthetic systems using cell walls or whole cells as a source of enzymes.

One or more transpeptidases are also implicated in cross bridge formation in these organisms, but transpeptidation has also never been achieved in vitro, except where cell walls replaced membranes as a source of enzymes (13, 14, 17).

This paper reports the detection of a membrane- and cell wall-associated *N*-acetylmuramyl-L-alanine amidase from *M. luteus* (*sodonensis*). This amidase functions in the absence of any detectable transpeptidase activity. Some evidence is presented to suggest that the enzyme may represent the amidase involved in peptidoglycan cross bridge formation.

MATERIALS AND METHODS

UDP-MurNAc-[^{14}C]pentapeptide. Uridine diphosphate-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine (UDP-MurNAc-[^{14}C]-pentapeptide) was prepared as previously described (10). Radioactivity was distributed among the three alanine residues of the peptide, and the purified material had a specific activity of 694 dpm/nmol. Analysis showed soluble *N*-acetylhexosamine, alanine, lysine, and glutamic acid to be present in a molar ratio of 1.0:3.2:1.04:0.99.

In vitro-synthesized peptidoglycan. In vitro-synthesized peptidoglycan was prepared and partially purified as previously described (10).

Membranes. Membranes were prepared from *M. luteus* (*sodonensis*) (ATCC 11880) grown in Trypticase soy broth (BBL, Cockeysville, Md.), pH 7.3, to the midexponential or stationary phase of growth. Cells were disrupted by short-term, high-speed mixing with plastic beads as previously described (10). Membrane fragments were collected by centrifugation, washed twice in 50 mM Tris-hydrochloride (pH 7.5) containing 1 mM β-mercaptoethanol (TME buffer), and resuspended to 7 mg of protein per ml for use.

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Crude cell walls. Whole cells were disrupted for cell wall isolation by short-term (2 min), high-speed mixing with plastic beads. Plastic beads and whole cells were removed by centrifugation at 1,000 × *g*. Cell walls were sedimented at 8,000 × *g*, washed twice in TME buffer, and resuspended to 1 mg of protein per ml for use.

Purified cell walls. Extensively purified cell walls, isolated from *M. luteus* (*sodonensis*) in the stationary phase of growth, were a gift from K. G. Johnson, National Research Council, Ottawa, Canada.

Enzyme assays. Transglycosidase assays were performed as described elsewhere (10). *N*-acetylmuramyl-L-alanine amidase activity was assayed by two methods.

(1) A standard transglycosidase assay was set up using stationary-phase membranes, incubated 3 h at 30 C, and boiled 1 min to stop the reaction. The amidase preparation to be tested was then added to the completed assay system. This amidase-containing mixture was incubated an additional 3 h at 30 C and again boiled 1 min. The stopped reaction mixture was streaked on a strip of Whatman 3 MM paper and chromatographed 16 h in solvent A. The dried strip was cut into 1-cm sections, and radioactivity was measured in a Nuclear-Chicago Mark I scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

(2) This assay was similar to (1), except that partially purified in vitro-synthesized peptidoglycan replaced the transglycosidase mixture as a source of substrate. The assay mixture also contained: 0.875 M Tris-hydrochloride, pH 8.6, 20 μl; 0.1 M $MgCl_2$, 10 μl. Final volume was 140 μl unless otherwise indicated, and the assay was incubated and developed in the same way as assay (1).

Analytical methods. Protein content of membrane suspensions and solubilized amidase preparations was measured by the method of Lowry et al. (12) after removal of β-mercaptoethanol by dialysis or dilution. Protein content of the cell wall suspensions was measured by a modification of the normal protein assay (16). Total hexosamines were determined by the modified Morgan-Elson technique (7) and soluble *N*-acetylhexosamines by the method of Strominger (20). Total amino acids were measured as their dinitrophenyl derivatives (7). N-terminal amino acids were measured by the technique described by Ghuysen et al. (7).

Chromatographic solvents. The chromatographic solvents were: solvent A, isobutyric acid-0.5 N aqueous ammonia (5:3, vol/vol) (3); solvent B, ethanol-1 M ammonium acetate (5:2, vol/vol) (3); solvent C, 1% aqueous ammonia-saturated butanol (7); and solvent D, chloroform-methanol-glacial acetic acid (85:14:1, vol/vol) (7).

RESULTS

Amidase activity of membranes from exponential-phase cells. Membrane suspensions prepared from *M. luteus* (*sodonensis*) cells in both the stationary and exponential phases of growth were compared for transglycosidase activity using the standard transglycosidase assay system. Unexpectedly, exponential-phase membranes appeared to have a much lower level of transglycosidase activity than stationary-phase membranes (only one-third as much radioactive peptidoglycan synthesized at the origin) (Fig. 1). Since peptidoglycan biosynthesis occurs most rapidly during cell growth and division, exponential-phase membranes were expected to contain higher levels of transglycosidase activity. As well as residual substrate (peak II, retardation factor [R_f] = 0.3) present in both systems, exponential phase-membrane chromatograms showed a new peak of radioactivity (peak III), the amidase product. This material was demonstrated by paper chromatography not to be free alanine or MurNAc-[^{14}C]pentapeptide and therefore was not the result of either transpeptidase, DD-carboxypeptidase, or phosphodiesterase activity (10). To investigate the possibility that the new material was the result of peptidoglycan breakdown, a type 1 amidase assay was performed. Exposure to exponential-phase membranes resulted in conversion of about 25% of the in vitro-synthesized peptidoglycan to the amidase product seen previously (Fig. 2). The apparently low level of transglycosidase activity observed in exponential-phase membranes was presumably due to the presence of this amidase, which was only present or active in exponential-phase membranes. Assuming that [^{14}C]pentapeptide

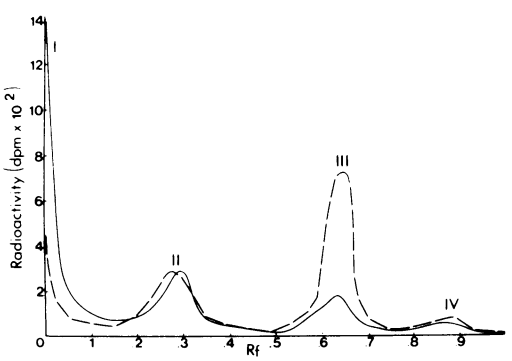

FIG. 1. *Transglycosidase and amidase activities in vitro in exponential- and stationary-phase membrane suspensions of M. luteus (sodonensis). The appropriate transglycosidase reaction mixtures were chromatographed on paper strips for 16 h in solvent A. Distribution of radioactive products was determined by cutting the strips into 1-cm sections and counting each section. Symbols: (——) Stationary-phase membranes; (- -) exponential-phase membranes. Peak I, In vitro-synthesized peptidoglycan; peak II, residual substrate (UDP-MurNac-[^{14}C]pentapeptide); peak III, amidase product ([^{14}C]pentapeptide); and peak IV, lipid precursors.*

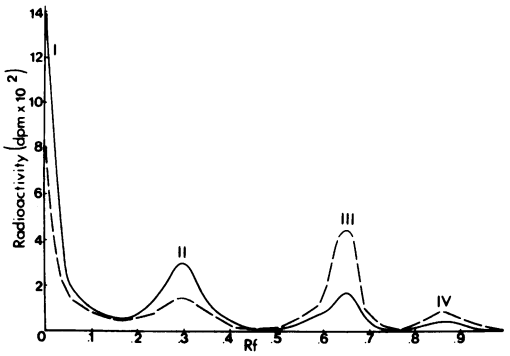

FIG. 2. *Measurement of amidase activity in membranes prepared from exponential-phase cells of M. luteus (sodonensis). In vitro-synthesized peptidoglycan was incubated with membranes prepared from exponential-phase cells. Reaction mixture was chromatographed and counted as for Fig. 1. Symbols: (——) Peptidoglycan control; (- -) peptidoglycan plus amidase containing exponential-phase membrane suspension. Peak I, In vitro-synthesized peptidoglycan; peak II, residual substrate (UDP-MurNac-[^{14}C]pentapeptide); peak III, amidase product ([^{14}C]pentapeptide); and peak IV, lipid precursors.*

can only arise from amidase digestion of in vitro-synthesized peptidoglycan, it became apparent that the rate of peptidoglycan synthesis was approximately equal for both exponential- and stationary-phase membrane preparations, but in the former case the peptidoglycan was subsequently being degraded by the amidase. The sum of radioactivity present as [^{14}C]pentapeptide and [^{14}C]peptidoglycan was, in fact, slightly greater for exponential-phase than for stationary-phase membrane preparations.

Identification of the amidase product. The product of this amidase activity was prepared for purification and analysis by scaling up a type 1 amidase assay 50-fold. After sedimenting out the membranes, the supernatant was applied to a Sephadex G-25 column (2.5 by 90 cm) and eluted with water. Radioactive fractions were pooled, lyophilized, and chromatographed on paper strips in solvent A. The amidase product (R_f = 0.65) was eluted and rechromatographed in solvent B. The active material was eluted, desalted again on the Sephadex G-25 column, and lyophilized. The purified amidase product was then assayed for soluble *N*-acetylhexosamine and total amino acids and shown to be free [^{14}C]pentapeptide (Table 1). The presence of glycine in the product indicated that some of the peptides had arisen due to amidase attack on preexisting cell wall peptidoglycan carried over by the membrane fragments. The UDP-MurNAc-[^{14}C]pentapeptide used in the in vitro synthesis of peptidoglycan did not contain glycine, but native cell wall peptidoglycan has glycine substituted on the α-carboxyl groups of glutamic acid in a mole-for-mole ratio (11). The presence of unlabeled peptides was also apparent from the decreased specific activity of the product peptide (500 dpm/nmol) when compared to the original substrate (694 dpm/nmol). The drop in the molar ratio of alanine, from 3.2 to 2.43, could also be explained if the unlabeled peptides arising from native peptidoglycan had only one C-terminal D-alanine residue rather than the D-alanyl-D-alanine dipeptide present in in vitro-synthesized peptidoglycan. The data suggest that the amidase product is a mixture of two peptides. Of the total 316 nmol of peptide, 226 nmol was derived from the in vitro-synthesized peptidoglycan and had a structure of [^{14}C]ala-glu-lys-[^{14}C]ala-[^{14}C]ala, whereas 90 nmol was derived from preexisting wall peptidoglycan with a structure of ala-isoglu(-gly)-lys-ala.

Hexosamine assays of in vitro-synthesized peptidoglycan fractions indicated the presence of large amounts of unlabeled peptidoglycan such that, overall, about two-thirds of the isolated peptidoglycan was not actually synthesized in vitro but came from unlabeled wall peptidoglycan (10). The composition of the amidase product, however, indicated that less than one-third of the peptides came from this unlabeled peptidoglycan. The amidase, therefore, preferentially degrades in in vitro-synthesized peptidoglycan over wall peptidoglycan.

Substrate specificity. The substrate specificity of the amidase indicated that it could only attack peptidoglycan. UDP-MurNAc-pentapeptide could not serve as a substrate, even though it contained the susceptible muramyl-L-alanine linkage. MurNAc-pentapeptide was also not acceptable as a substrate, which indicated that the nucleotide portion was not responsible for the inability to use UDP-MurNAc-pentapeptide.

In an analogous experiment, the in vitro-synthesized peptidoglycan was exposed to lyso-

TABLE 1. *Composition of the product of M. luteus (sodonensis) amidase on autogenous in vitro-synthesized peptidoglycan*

Component	Amount[a]	Molar ratio
Soluble *N*-acetylhexosamine	0	
Glutamic acid	322	1.00
Glycine	90	0.30
Alanine	784	2.43
Lysine	310	0.96

[a] Nanomoles per milliliter.

zyme and completely degraded into disaccharide- and tetrasaccharide-peptide fragments. Amidase-containing, exponential-phase membranes were then added to the lysozyme digest, but they were unable to use either disaccharide- or tetrasaccharide-peptide as substrate (Fig. 3). A peak of residual UDP-MurNAc-[^{14}C]pentapeptide allows the exponential-phase membranes to form some free pentapeptide through transglycosidation and subsequent degradation, but there is no change in the amount of the two lysozyme digest products upon exposure to exponential-phase membranes. The low-molecular-weight species of the in vitro-synthesized peptidoglycan (10) with an estimated chain length of 20 disaccharide-peptide units was degraded by amidase. This indicates that the amidase has a requirement for peptidoglycan chains of some minimum size, more than 2 disaccharide-peptide units (tetrasaccharide-peptide) but less than 20 disaccharide-peptide units.

Cellular location and function of the amidase. Crude cell wall preparations isolated from exponential-phase cells were found to have high levels of amidase activity. Using a type 1 amidase assay, exponential-phase cell walls released 23.8 nmol of [^{14}C]pentapeptide per h per mg of protein, whereas exponential-phase membranes released 2.0 nmol of [^{14}C]pentapeptide per h per mg of protein. The majority of protein in a crude cell wall preparation is due to the presence of membrane fragments attached in some manner to the cell wall structure. The large difference in specific activity of membrane- versus cell wall-bound amidases indicates that the amidase activity of crude cell wall preparations is somehow different and not just due to the presence of membrane fragments. In contrast, exponential-phase membrane and cell wall preparations had roughly equivalent amounts of transglycosidase activity based on protein content.

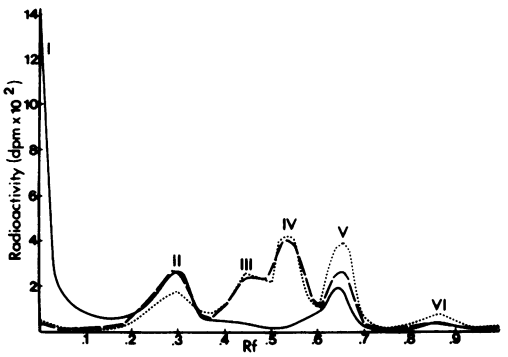

FIG. 3. *Effect of lysozyme digestion on the susceptibility of in vitro-synthesized peptidoglycan to amidase activity of membranes prepared from exponential phase cells of M. luteus (sodonensis). Peptidoglycan was synthesized in vitro by stationary phase membrane suspensions of M. luteus (sodonensis), digested completely with lysozyme, and then exposed to membrane-bound amidase. Reaction mixture was chromatographed and counted as described for Fig. 1. Symbols: (——) Peptidoglycan control; (— —) peptidoglycan plus lysozyme; (-----) peptidoglycan plus lysozyme plus amidase containing exponential phase membrane suspension. Peak I, in vitro-synthesized peptidoglycan; peak II, residual substrate (UDP-MurNac-[^{14}C]pentapeptide); peaks III and IV, tetrasaccharide and disaccharide peptides, respectively (lysozyme products); peak V, amidase product ([^{14}C]pentapeptide); and peak VI, lipid-associated material.*

Neither exponential-phase membranes nor cell walls showed any evidence of transpeptidase activity when assayed in a standard transglycosidase system. In an attempt to stimulate transpeptidase activity, the assay system was supplemented with adenosine 5'-triphosphate, glycine, and NH_4Cl and buffered to a pH of 7.8. Using this reaction mixture, Mirelman had demonstrated transpeptidase activity in cell wall suspensions of *M. luteus* (*lysodeikticus*) (14), but no such activity could be detected in *M. luteus* (*sodonensis*) membranes or in cell walls. Furthermore, variations in the method of disrupting cells to obtain membrane and wall preparations did not yield preparations with demonstrable transpeptidase activity.

To determine whether the amidase of *M. luteus* (*sodonensis*) functions as an autolysin, exponential- and stationary-phase whole cells were harvested, washed in TME buffer at 4 C, and then suspended in TME buffer to an absorbancy of 0.5 at 600 nm. The cell suspensions were incubated at 30 C and changes in absorbancy at 600 nm were followed. Both cell suspensions were incubated at 30 C and changes in absorbancy at 600 nm were followed. Both cell suspensions were equally resistant to autolysis, even though the exponential-phase cell preparations contain high levels of amidase, whereas those of stationary-phase cells contain none. This lack of correlation between presence of amidase and autolysis seems to argue against such a role for this enzyme in vivo.

Purification of amidase. Three methods of solubilization involving extraction with Triton X-100 (23), with butanol (2), and with LiCl (18) were used to solubilize amidase activity from membrane suspensions (7 mg of protein per ml) and cell wall suspensions (1 mg of protein per ml). Details of each solubilization were given previously (10). The residual pellets of membranes and cell walls from these three methods were resuspended to their original volumes in

TME buffer. The resuspended pellets and aqueous extracts were all dialyzed 16 h against TME buffer before amidase activity was measured. When protein content was to be measured, TME buffer lacking β-mercaptoethanol was used for dialysis. A 50-μl portion of each amidase preparation was assayed for activity in a type 1 amidase assay.

All three methods successfully solubilized amidase activity from both membranes and cell walls, and in every case total activity recovered was more than 100% (Table 2). LiCl extraction was the most successful method because of the low levels of contaminating protein that were solubilized. LiCl extracts were also functionally pure since neither transglycosidase nor phosphodiesterase activities will withstand LiCl extraction (10).

To compare the properties of cell wall- and membrane-derived amidases, cell walls and membranes were prepared from a 2.4-liter batch of exponential-phase cells. Amidase activity was solubilized from each of these preparations using LiCl extraction. The dialyzed extracts from each were concentrated to 2.5 ml by lyophilization and applied to a Sephadex G-200 column (2.5 by 45 cm), which was equilibrated and eluted with TME buffer (Fig. 4). Five-milliliter fractions were collected, and absorbancy at 280 nm was measured. A 100-μl portion of each positive fraction was then assayed for amidase activity using a type 1 amidase assay. Disappearance of radioactivity from the origin of the chromatograms was used as a measure of activity. Both cell wall- and membrane-derived amidase activities eluted in the void volume of the column, indicating that the enzyme or enzyme-containing complex solubilized was of a high molecular weight (greater than 150,000). The amidase-containing fractions from both Sephadex G-200 columns were concentrated to 5 ml by ultrafiltration using an Amicon PM-10 filter. The membrane amidase concentrate contained 0.267 mg of protein per ml, and the cell wall amidase concentrate contained 0.056 mg of protein per ml.

Amidase digestion of purified cell walls. The partially purified cell wall- and membrane-derived amidases were tested for their ability to degrade purified cell walls of *M. luteus (sodonensis)* (Table 3). By analysis, the 0.7-mg amount of purified cell walls used as substrate contained about 50 nmol of the susceptible *N*-acetylmuramyl-L-alanine amidase linkage and no detectable free N-terminal alanine (K. G. Johnson, Ph.D. thesis, University of Alberta, Edmonton, Alberta, 1971). The 1.0-ml amount of each amidase preparation was chosen as an amount capable of releasing in excess of 50 nmol of free [^{14}C]pentapeptide from the in vitro-synthesized peptidoglycan, as measured in a type 1 amidase assay. The membrane-derived, partially purified amidase preparation was, however, found to have lost activity upon freezing at −70 C. A 1.0-ml portion of this preparation was capable of causing the release of only 25 nmol of free [^{14}C]pentapeptide from in vitro-synthesized peptidoglycan.

The observed release of 53.4 nmol of N-terminal alanine by cell wall-derived amidase and 24.2 nmol of N-terminal alanine by membrane-derived amidase indicated that solubilized amidases can attack cross-linked cell wall peptidoglycan in addition to the in vitro-synthesized peptidoglycan. Membrane- and cell wall-bound amidases caused no release of N-terminal alanine above background.

Amidase digestion of in vitro-synthesized peptidoglycan fractions. The ability of both the soluble and bound forms of exponential-phase membrane and cell wall amidase to attack fractionated, in vitro-synthesized peptidoglycan was examined (Table 4). The peptidoglycan fractions were the high- and low-molecular-weight species of "soluble" and "insoluble" peptidoglycan, as separated by Sephadex G-200 chromatography (10). Each of the four types of peptidoglycan was digested with both the solubilized and bound forms of cell wall- and membrane-derived amidase using a type 2 amidase assay. A 900-dpm portion of each type of peptidoglycan was used per assay. Initially, the

TABLE 2. *Solubilization of amidase from exponential-phase membrane and cell wall suspensions of M. luteus (sodonensis)*

Solubilization techniques	Membrane suspensions			Cell wall suspensions		
	Amidase solubilized[a]	Amidase remaining bound[a]	Sp act of soluble extract[b]	Amidase solubilized[a]	Amidase remaining bound[a]	Sp act of soluble extract[b]
Triton X-100 extraction	82.7	63.3	3.50	82.0	88.9	24.9
Butanol extraction	56.1	76.0	4.20	83.4	88.9	39.6
LiCl extraction	60.0	42.0	24.0	77.5	85.7	108.5

[a] Percentage of total original amidase activity.

[b] Nanomoles of [^{14}C]pentapeptide produced per hour per milligram of protein.

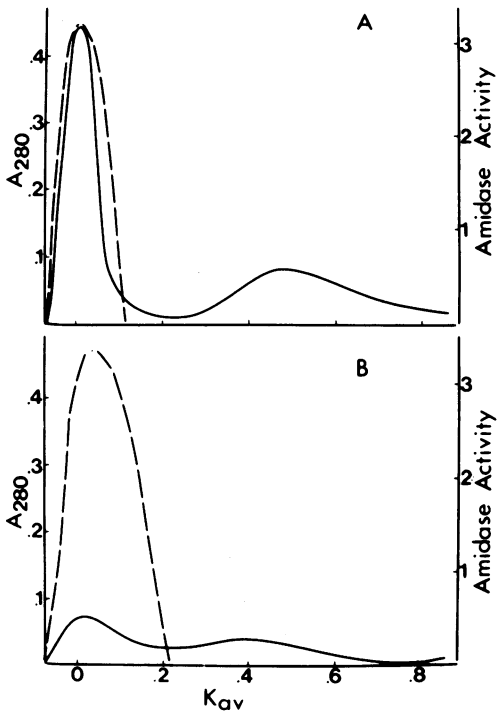

FIG. 4. *Partial purification of solubilized membrane- and cell wall-derived amidase of M. luteus (sodonensis) by Sephadex G-200 filtration. (A) Membrane-derived amidase. (B) Cell wall-derived amidase. Amidase activity, solubilized from exponential-phase membranes and cell walls by LiCl extraction as described in Materials and Methods, were applied separately to a Sephadex G-200 column (2.5 by 45 cm), equilibrated, and eluted with TME buffer. Protein and amidase activity of effluent fractions were monitored by absorbancy at 280 nm and a type 1 amidase assay, respectively. Symbols: (——) Protein (A_{280}); (– –) amidase activity (nanomoles of pentapeptide released per milliliter of enzyme).*

amounts of exponential-phase membranes, exponential-phase cell walls, partially purified membrane-derived amidase, and partially purified cell wall-derived amidase required to degrade from 50 to 60% of high-molecular-weight soluble peptidoglycan was determined. A 50-μl portion of membrane suspension (3.5 mg of protein per ml), a 50-μl portion of membrane-derived amidase (0.089 mg of protein per ml), a 50-μl portion of cell wall suspension (0.04 mg of protein per ml), and a 50-μl portion of cell wall-derived amidase (0.007 mg of protein per ml) were found to be the appropriate amounts and dilutions. These amounts were then assayed for their ability to degrade the other three peptidoglycan types. The amount of radioactivity released from the labeled peptidoglycan relative to a control was used as a measure of amidase activity. The amidase activity against high-molecular-weight soluble peptidoglycan was arbitrarily assigned a value of 100, and activity against the other three peptidoglycan species was related to that value. Membrane and cell wall amidases, both solubilized and bound, differed in their abilities to attach these different peptidoglycan types. The high-molecular-weight soluble peptidoglycan was the most acceptable substrate for all amidase preparations. Cell wall-bound amidase exhibited a significantly reduced affinity for the low-molecular-weight peptidoglycan, especially the soluble variety (30% of maximum). The amidase solubilized from membranes had a somewhat more reduced affinity for the high-molecular-weight insoluble peptidoglycan than did its membrane-bound counterpart. Both

TABLE 3. *Digestion of purified cell walls of M. luteus (sodonensis) by autogenous amidase*[a]

Amidase source	N-terminal alanine released in test (substrate added) (nmol)	N-terminal alanine released in control (no substrate added) (nmol)	N-terminal alanine specifically released by added amidase (nmol)
LiCl extract of membranes	83.6	59.4	24.2
LiCl extract of cell walls	60.8	7.4	53.4
Exponential-phase membranes	46.3	46.9	0
Exponential-phase cell walls	111.6	109.7	1.9

[a] Incubation mixtures were set up containing 0.7 mg of highly purified cell walls in 70 μl of water; 0.875 M Tris-hydrochloride, pH 8.6, 0.4 ml; and 0.1 M $MgCl_2$, 0.2 ml. One-milliliter volumes of each of four types of amidase were assayed: partially purified cell wall-derived amidase (0.056 mg of protein per ml), partially purified membrane-derived amidase (0.267 mg of protein per ml), exponential-phase cell walls (1 mg of protein per ml), and exponential-phase membranes (7 mg of protein per ml). For each of the four types of amidase, a control incubation mixture was set up omitting the cell walls. The assays and controls were incubated 3 h at 30 and then boiled for 1 min to stop the reaction. Assays and controls were then centrifuged 45 min at 48,000 $\times$ *g*, and the supernatants were desalted on a calibrated Sephadex G-15 column (1.5 by 30 cm). All fractions from the void volume to the K_{AV} (reference 10) of 0.85 (where the salt begins to elute) were pooled and lyophilized. The dried material was resuspended in 200 μl of distilled water and assayed for N-terminal alanine.

TABLE 4. *Digestion of fractionated in vitro-synthesized peptidoglycan by amidase*

Substrate	Relative amidase activity[a]			
	Solubilized membrane amidase	Membrane-bound amidase	Solubilized cell wall amidase	Cell wall-bound amidase
High-molecular-weight soluble peptidoglycan	100[a]	100[a]	100[a]	100[a]
Low-molecular-weight soluble peptidoglycan	81	71	67	30
High-molecular-weight insoluble peptidoglycan	51	83	80	75
Low-molecular-weight insoluble peptidoglycan	87	82	87	59

[a] Amidase activity against a high-molecular-weight soluble peptidoglycan was arbitrarily assigned a value of 100, and activity against the other three peptidoglycan species was expressed relative to that.

membrane-derived amidase preparations were more capable of digesting the low-molecular-weight peptidoglycan than their respective wall-derived counterparts.

DISCUSSION

The amidase activity detected in exponential-phase membranes and cell walls of *M. luteus* (*sodonensis*) would appear to be a synthetic enzyme involved in cross bridge formation. Such a function would be consistent with its presence in exponential-phase cells only, its preference for in vitro-synthesized, uncross-linked peptidoglycan, and its lack of autolytic characteristics. Since transpeptidation does not take place in the in vitro system used, the release of free pentapeptide may be the result of a normally synthetic amidase activity functioning in the absence of transpeptidase activity. The substituent peptides, cleaved by the amidase, are released into the medium rather than being incorporated into bridge peptides. This is consistent with a previous finding that free pentapeptide is detectable in the spent culture medium of *M. luteus* (*sodonensis*) when growing in the presence of penicillin (unpublished observation). Penicillin would inhibit transpeptidation, whereas amidase activity would continue unabated.

Transglycosidase activity is a membrane-bound enzyme system, and its detection in cell wall preparations is due to the presence of membrane material attached to the wall fragments. Amidase activity, on the other hand, is present in membrane preparations but predominates in cell wall preparations. The enzyme may be located at the wall-membrane interface which, upon disruption, exhibits a stronger tendency to associate with cell wall material. Alternatively, there may be two separate forms of amidase, one membrane-bound and one cell wall-bound. Certainly, there is a major difference in specific activity between the membrane- and cell wall-bound forms of amidase. As well, the membrane- and cell wall-bound forms of amidase differ in their ability to degrade the various fractions of the in vitro-synthesized peptidoglycan. The low-molecular-weight material, which makes up about 70% of the total peptidoglycan, is only poorly degraded by cell wall amidase, whereas membrane amidase is better able to attack it. An amidase functioning in cross bridge formation would have to use this low-molecular-weight peptidoglycan to incorporate it into pre-existing cell walls. The ability of exponential-phase cell walls to degrade the added in vitro-synthesized peptidoglycan, even though the cell walls themselves contain large amounts of unlabeled peptidoglycan, indicates that the amidase has a preference for the highly soluble, uncross-linked, in vitro-synthesized material.

Evidence of peptidoglycan lytic activity has been reported in cell-free systems of *Escherichia coli* (9). *E. coli* is known to possess a wide range of membrane-bound peptidoglycan-hydrolyzing enzymes, one of which is an *N*-acetylmuramyl-L-alanine amidase (8). The only activity detected in the cell-free system was an *N*-acetylmuramidase. Unlike *E. coli*, gram-positive microorganisms most frequently contain autolysins in their cell walls rather than membranes. *Staphylococcus aureus* contains several types of cell wall-bound autolytic enzymes including an *N*-acetylmuramyl-L-alanine amidase (21), but no evidence of this activity has been detected in cell-free peptidoglycan-synthesizing systems (1). The amidase activity of *M. luteus* (*sodonensis*), therefore, differs from those of the above-mentioned organisms and may represent a synthetic activity rather than an autolytic one.

ACKNOWLEDGMENTS

We wish to thank Lucille M. Phillippe for her technical assistance.

This work was supported by the National Research Council of Canada (operating grant A-1743).

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