Novel Type of Murein Transglycosylase in *Escherichia coli*

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The purification and properties of a novel type of murein transglycosylase
from *Escherichia coli* are described. The purified enzyme appears as a single band
on sodium dodecyl sulfate-polyacrylamide gels and has an apparent molecular
weight of approximately 65,000 as estimated by gel filtration and gel electropho-
resis. It degrades pure murein sacculi from *E. coli* almost completely into
low-molecular-weight products. The two prominent muropeptide fragments in
the digest are the disaccharide-tripeptide *N*-acetylglucosamine–*N*-acetylmu-
ramic acid-L-alanine-D-iso-glutamic acid-*meso*-diaminopimelic acid and the
corresponding disaccharide-tetrapeptide *N*-acetylglucosamine–*N*-acetylmuramic
acid-L-alanine-D-*iso*-glutamic acid-*meso*-diaminopimelic acid-D-alanine. The
unique feature of these compounds is that the disaccharide has no reducing end
group and that the muramic acid residue possesses an internal 1 → 6 anhydro
linkage. The new lytic enzyme is designated as a murein:murein transglycosylase.
Its possible role in the rearrangement of murein during cell growth and division is
discussed.

The morphological changes of a bacterial cell
during growth and division are accompanied by
modifications of the murein sacculus. This
shape-maintaining macromolecule completely
encloses the cell. Since the sacculus is held
together by covalent bonds, any alteration in
shape must involve breaking of at least some of
the preexisting bonds. Therefore, it has been
proposed by several authors that murein hydro-
lases, in concert with synthesizing enzymes,
may play an essential role in the biosynthesis
and assembly of the murein sacculus (4, 21, 28).

Escherichia coli has been shown to contain
different murein-degrading enzymes (4, 16, 28)
whose specific function in bacterial morpho-
genesis is still obscure. Detailed information
on the properties of the individual enzymes
may lead to a better understanding of their
biological role.

A novel type of lytic enzyme that degrades
the cell wall murein into low-molecular-weight
muropeptides lacking reducing end groups has
been found in *E. coli* (7, 9). In this communica-
tion we describe the purification and properties
of this unique murein-degrading enzyme and
provide evidence on the chemical structure of
its reaction product(s). We show that this
enzyme converts the glycosidic bond between
N-acetylmuramic acid and *N*-acetylglucosa-
mine into an intramolecular 1 → 6 anydro-*N*-
acetylmuramyl bond. The enzyme can, there-
fore, be classified as transglycosylase. The pos-
sible biological function of the enzyme is dis-
cussed.

MATERIALS AND METHODS

Lytic activity was routinely determined by meas-
uring the release of radioactivity from sacculi labeled
with tritiated 2,6-diaminopimelic acid. Under stan-
dard conditions, 25 µl of [^{3}H]diaminopimelic acid-
labeled sacculi (2 × 10^6 counts/min per ml, 0.5 mg of
murein/ml) was incubated with 25 µl of enzyme and
150 µl sodium acetate buffer (0.4 M; pH 4.5) contain-
ing 0.2% (wt/vol) Triton X-100 for 10 min at 37 C. The
reaction was stopped by putting the samples in ice
and adding 20 µl of bovine serum albumin (5%,
wt/vol) followed by 20 µl of ice-cold trichloroacetic
acid (50%, wt/vol). The precipitate was sedimented
(Eppendorf centrifuge, 2 min at 12,000 × g), 100 µl
of the supernatant was diluted with 0.5 ml of 0.1 N
NaOH, and the radioactivity was measured as de-
scribed below.

One unit of lytic activity is defined as the amount
of enzyme that solubilizes 1 µg of murein under the
standard conditions in 10 min at 37 C.

Enzyme purification. *E. coli* W7 (7) was grown in
a minimal medium (26) at 37 C to a cell density of 5 ×
10^8/ml. Cells were collected by centrifugation and
washed once with cold phosphate buffer (0.01 M, pH
6.9). All subsequent steps were done at 2 to 4 C.

(i) **Cell extract.** A suspension of 140 g (wet weight)
of cells in 280 ml of phosphate buffer (0.01 M; pH 6.9)
was shaken in portions of 80 ml with 240 g of glass
beads (0.17 to 0.18 mm diameter; Braun, Melsungen,

Germany) and 4 μg of deoxyribonuclease per ml in a cell mill (Bühler, Tübingen, Germany). The glass beads were removed by filtration and washed with 500 ml of buffer, and the combined filtrate was clarified by centrifugation (40 min at 48,000 × g). The supernatant (740 ml) was then divided into two portions for further fractionation (steps ii and iii). Before application to the first diethylaminoethyl (DEAE)-cellulose column (step iv), the fractions were recombined.

(ii) Ammonium sulfate precipitation. The supernatant (370 ml; 9.1 mg of protein per ml; 5.4 U/mg) was diluted with 0.01 M phosphate buffer (pH 6.9) to a final concentration of about 3 mg of protein per ml. Ammonium sulfate was slowly added under constant stirring to bring the solution to 30% saturation. The solution was kept at 0 C for 12 h, and the precipitate was removed by centrifugation (20 min at 15,000 × g). The clear supernatant (1,200 ml; 1.87 mg of protein per ml) was brought to 50% saturation with ammonium sulfate. After 12 h, the precipitate was collected by centrifugation (30 min at 12,000 × g) and resuspended in phosphate buffer (0.01 M; pH 6.9), resulting in 45 ml of a solution with 18.4 mg of protein per ml and 10.2 U/mg.

(iii) Gel filtration. After dialysis of the solution from step ii against 3 liters of 20% saturated ammonium sulfate in 0.1 M phosphate buffer (pH 6.9), the enzyme was applied to an agarose column (Bio-Gel A-1.5 m; 68 by 5 cm). The column, equilibrated with the dialysis solution, was eluted with the same solution at a flow rate of 57 ml/h. Fractions of 9.5 ml were collected and tested for protein and lytic activity. The fractions with enzyme activity (tubes 103 through 117) from two preparations were combined and dialyzed twice against 5 liters of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) to yield 296 ml of solution (0.77 mg of protein per ml, 50 U/mg).

(iv) First DEAE-cellulose chromatography. The solution from step iii was applied to a column (15 by 2 cm) of DEAE-cellulose (Whatman DE 32) that had been equilibrated with 0.01 M Tris-hydrochloride buffer (pH 8.0). Elution was with a linear gradient of NaCl (mixing chamber—100 ml of 0.03 M NaCl in 0.01 M Tris-hydrochloride buffer, pH 8.0; reservoir—100 ml of 0.3 M NaCl in the same buffer). Fractions of 1.2 ml were collected at a flow rate of 11 ml/h. The enzyme was eluted between 0.04 and 0.08 M NaCl (See Fig. 2A). Fractions 34 to 60 from the column were pooled and dialyzed twice against 4 liters of 0.01 M phosphate buffer (pH 6.9) for 24 h. The dialyzed enzyme (50 ml; 0.20 mg of protein per ml) had a specific activity of 1,010 U/mg.

(v) First hydroxylapatite chromatography. A column (37 by 1 cm) of hydroxylapatite (Bio-Rad HTP) was thoroughly washed with 0.01 M phosphate buffer, pH 6.9. The enzyme from step iv was applied to the column and eluted with a linear phosphate buffer gradient (mixing chamber—100 ml of 0.05 M phosphate, pH 6.9; reservoir—100 ml of 0.2 M phosphate, pH 6.9) at a flow rate of 11 ml/h. Fractions of 1.1 ml were collected and tested for lytic activity, which was eluted in fractions 94 to 120 (0.04 to 0.09 M phosphate buffer) (Fig. 2B). After dialysis twice

against 3 liters of 0.01 M Tris-hydrochloride buffer (pH 8.0), the preparation (34 ml; 0.085 mg of protein per ml) had a specific activity of 3,240 U/mg.

(vi) Second DEAE-cellulose chromatography. The enzyme from step v was purified further on a second DEAE-cellulose column (20 by 1 cm). Elution with a linear NaCl gradient (mixing chamber—100 ml of 0.02 M NaCl; reservoir—100 ml of 0.5 M NaCl; both in 0.01 M Tris-hydrochloride buffer, pH 8.0). Fractions of 0.9 ml were collected at a rate of 9 ml/h. Fractions 23 to 35 containing enzyme activity were pooled and dialyzed against 5 liters of 0.01 M phosphate buffer (pH 6.9) for 12 h. This preparation (11 ml) contained 0.048 mg of protein per ml and had a specific activity of 4,720 U/mg.

(vii) Second hydroxylapatite chromatography. Final purification was achieved by chromatography on a column of hydroxylapatite (28 by 0.9 cm) with a linear phosphate buffer gradient (mixing chamber—100 ml, 0.04 M; reservoir—100 ml, 0.2 M, both at pH 6.9). Elution was at 4.5 ml/h and the fraction size was 0.6 ml. Fractions with enzyme activity (between 33 and 45 ml) were combined (12 ml; 1.5 μg of protein per ml, 7,670 U/ml) and stored at 1 C. In later experiments, smaller columns (0.5 by 6 cm) were found to give better recovery of active enzyme.

Large-scale preparation of reaction products. Sacculi were prepared on a large scale essentially as described by Mardarowicz (13). *E. coli* W7 cells (500 g wet weight), suspended in distilled water (500 ml), were added slowly into 2 liters of a boiling solution of sodium dodecyl sulfate (4%, wt/vol) under vigorous stirring. The mixture was kept boiling for 30 min after which the sacculi were collected by centrifugation (45 min; 27,000 × g). The sacculi were purified further by digestion with α-amylase (10) and Pronase (2) to yield 1.13 g of lyophilized material. The purified sacculi (330 mg), containing a small amount of [³H]diaminopimelic acid-labeled sacculi (10^7 counts/min) as a tracer, were suspended in 470 ml of buffer (10 mM sodium acetate, pH 4.5; 10 mM Mg^{2+}; 0.05% bovine serum albumin) with 5,600 U of purified enzyme (dissolved in 140 ml of incubation buffer). After incubation at 37 C for 20 h, the reaction was stopped by boiling in a water bath (5 min). Protein was sedimented by low-speed centrifugation (10 min; 2,000 × g), and the supernatant was lyophilized. The residue was dissolved in 5 ml of water and desalted on a column of Sephadex G 25 (2.7 by 45 cm; elution with water, 22 ml/h; fractions of 3 ml). Two radioactive peaks (fractions 20 through 30 and 31 through 38) were obtained, each of which was lyophilized and analyzed by paper chromatography in solvent I (see below). The first peak, accounting for about 20% of the radioactivity applied to the column, contained material that did not migrate on paper and was therefore discarded. The second peak (R_{C6} 1.4; muropeptide C6 as a reference) was purified further by chromatography on DEAE-cellulose. The column (2.7 by 45 cm) was equilibrated with 10 mM Tris-hydrochloride (pH 7.2) and eluted with a linear salt gradient (mixing chamber—400 ml of equilibration buffer; reservoir—400 ml of 10 mM equilibration buffer containing 0.3 M NaCl). Elution rate was at 44

ml/h; radioactive material appeared in a single sharp peak at 0.08 M NaCl. The peak fractions were pooled, lyophilized, and again desalted on a Sephadex G 25 column as described above. Paper chromatography in solvent I (see below) revealed a mixture of two compounds, designated as X and X', that had R_{C_6} values of 1.27 and 1.52, respectively, when muropeptide C6 (17) was taken as a reference. The total amount of X and X' obtained was about 40 mg. A portion of the X/X' mixture was fractionated further into X and X' by preparative paper chromatography in solvent I.

Molecular weight. The molecular weight of the enzyme was determined by gel filtration on agarose (Bio-Gel A-1.5 m) and on polyacrylamide (Bio-Rad P-150). Blue dextran was not applied simultaneously with the enzyme; the dye causes a shift in the elution volume of the enzyme, the two being then eluted together in the void volume of the column. The columns were eluted, by the method of Andrews (1), with 0.1 M KCl in 0.01 M phosphate buffer, pH 6.9. Proteins of known molecular weight were used as standards, and the V_e/V_o values were plotted versus the logarithm of the molecular weight.

Analytical procedures. Radioactivity in aqueous samples was measured in a Triton-toluene scintillation fluid (6); counting efficiency was 30% for tritium and 74% for ^{14}C. Radioactivity on paper was detected with a Packard radiochromatogram scanner (model 3200) and quantitated in a standard toluene-based scintillator (5 g of 2,5-diphenyloxazole per liter of toluene; counting efficiency, 82% for ^{14}C). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed at room temperature with 5% gels (0.5 by 9 cm) in 0.1% sodium dodecyl sulfate (pH 7.2) by the method of Weber and Osborn (27).

Protein was assayed by the method of Lowry et al. (11), with egg white lysozyme (crystallized three times; Merck, Darmstadt, Germany) as a standard.

Descending paper chromatograms were run on Whatman 3 MM or Schleicher and Schüll 2043b-Mgl paper with solvent I (n-butanol-acetic acid-water, 4:1:5; vol/vol, upper phase) for 48 or 72 h. Paper electrophoresis was on Whatman 3MM paper at pH 3.5 (0.2 M acetic acid brought to pH 3.5 with dilute pyridine) or pH 6.5 (0.2 M pyridine brought to pH 6.5 with dilute acetic acid) at 50 V/cm for 70 min.

Reducing groups (15), N-acetyl amino sugars (18), and O-acetyl groups (8) were determined colorimetrically. Reducing compounds were detected on paper chromatograms with silver nitrate reagent (22), and N-acetyl amino sugars were detected by fluorescence (23). Analysis of hydrolysates (6 M HCl at 105 C for 18 h in sealed tubes) was done on a Beckman 120 C amino acid analyzer (14). Radioactivity in the hydrolysates was monitored by passing the effluent from the amino acid analyzer column directly through a flow scintillation spectrometer (Packard Tri-Carb 3200).

Dinitrophenylation studies were carried out as described previously (14). Reduction with NaB[^{3}H$_4$] (244 mCi/mmol) was done by the procedure of McLean et al. (12).

Digestion of muropeptides (50 µg) with Strep-tomyces amidase was as described previously (3). Digestion of muropeptides (50 µg) with a crude Charonia lampas glycosidase preparation (Seikagaku Kogyo Co., Tokyo) was carried out at a concentration of 1 mg of enzyme per ml in citrate buffer (pH 4.6; 0.05 M) for 18 h at 37 C. For acetylation of muropeptides or N-acetyl-D-glucosamine, 0.7 to 0.8 mg of material was suspended in anhydrous pyridine (100 µl) to which was added [1-^{14}C]acetic anhydride (25 µl) that had been premixed with unlabeled acetic anhydride to yield a specific radioactivity of 0.1 µCi/µmol. The reaction mixture was kept at 37 C for 72 h with occasional mixing, after which the solution was evaporated in vacuo. Toluene (2 ml) was added and evaporated, and this procedure was repeated two more times after which three dissolutions and evaporations with methanol (2 ml each) were carried out. The reaction mixture was purified further by descending paper chromatography (Whatman 3MM; 20 h in solvent I). The radioactivity on the chromatograms was detected by exposure to an X-ray film (Kodak RP-54). The peracetylated muropeptides do not migrate on the chromatograms, and the radioactive material remaining at the origin was eluted from the paper with methanol. Aliquots of the radioactive materials were counted.

For analysis of amino sugars (14) and amino acids, a portion of the eluted materials (75% of the radioactivity) was evaporated to dryness in a tube and 6 N HCl (1 ml) was added. The tube was sealed, hydrolysis was carried out for 16 h at 100 C, the acid was evaporated in vacuo, and the hydrolysate was analyzed quantitatively.

Crystalline 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-glucose (melting point, 130 C), a gift from A. Liav, was used as a marker for chromatography of the radioactively acetylated N-acetyl-D-glucosamine. The acetylated compound was detected by the method of Hestrin (8); its rate of migration on the chromatogram was identical to that of the radioactively acetylated derivative. Analysis of the radioactive fully acetylated N-acetyl-D-glucosamine was as described above for the muropeptides.

RESULTS AND DISCUSSION

Purification of the enzyme. E. coli contains a number of murein hydrolases that are known to be tightly bound to the cell envelope (for review see references 4, 5, and 6). Our sonic oscillation procedure leads to preferential release of the murein transglycosylase, which is only loosely bound to the envelope (E. W. Goodell, unpublished data).

The novel transglycosylase from E. coli, which degrades murein into nonreducing, low-molecular-weight fragments, could be purified 1,400-fold by a six-step procedure. A representative preparation scheme is described in detail above and is summarized in Table 1.

About half of the murein hydrolase activity of the crude cell extract was recovered by precipi-

TABLE 1. Summary of recoveries in the purification steps of the transglycosylase[a]

Purification step	Total vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Enzyme concn (U/ml)	Total enzyme activity (U)	Sp act (U/mg)	Yield of enzyme (%)	Purification factor
Crude extract	740	9.1	6,734	49	36,364	5.4	100	
Ammonium sulfate precipitation	90	18.4	1,656	187	16,891	10.2	46.5	1.9
Agarose (Bio-Gel A-1.5 m) column	296	0.77	228	39	11,510	50.5	31.7	9.4
First DEAE column (I)	50	0.204	10.4	206	10,508	1,010	29.0	187.0
First HAP[b] column (I)	34	0.085	2.93	275	9,501	3,240	26.1	600.0
Second DEAE column (II)	11	0.048	0.528	226	2,492	4,720	6.8	875
Second HAP column (II)	12	0.0015	0.018	11.5	138	7,670	0.4	1,420

[a] One unit of transglycosylase is defined as the amount of enzyme that solubilizes 1 μg of labeled murein under standard assay conditions after 10 min at 37 C.

[b] HAP, Hydroxylapatite.

tation with ammonium sulfate between 30 and 50% saturation. Depending on the ionic conditions, the redissolved precipitate was eluted as aggregates of differing sizes from an agarose column. However, the enzyme could be obtained in an exclusively low-molecular-weight form when the precipitate was dissolved and eluted with a phosphate buffer containing ammonium sulfate at 20% saturation (Fig. 1). Under these conditions, good separation of the enzyme from other proteins was achieved, resulting in a ten-fold purification.

After chromatography on DEAE-cellulose (Fig. 2A), the eluate did not show any enzyme activity which, however, could be restored by the addition of 0.05% serum albumin or of 0.2% Triton X-100 to the fractions. Enzyme assays were routinely carried out with 0.2% Triton X-100 in the reaction mixture.

The purified enzyme obtained from step vi appeared as a single band upon sodium dodecyl sulfate-gel electrophoresis (Fig. 3), migrating at the same rate as bovine serum albumin, which indicated an apparent molecular weight of 65,000. This figure agrees with the values found by gel filtration: the experimental V_e/V_o values on Bio-Rad P-150 and on Bio-Gel A-1.5 m corresponded to apparent molecular weights of 59,000 and 58,000, respectively.

Properties of the purified enzyme. The optimal pH for the activity of the purified enzyme is 4.5 in sodium acetate buffer. The activity at pH 3.5 and 5.5 was 55 to 60% of that at pH 4.5. Table 2 summarizes the effect of several mono- and divalent cations on the activity of the enzyme. Although the overall ionic strength affects enzyme activity, no specific ion effect was found. Optimal ionic conditions were obtained either by adding 0.05 M MgCl to a 0.01 M sodium acetate buffer or by
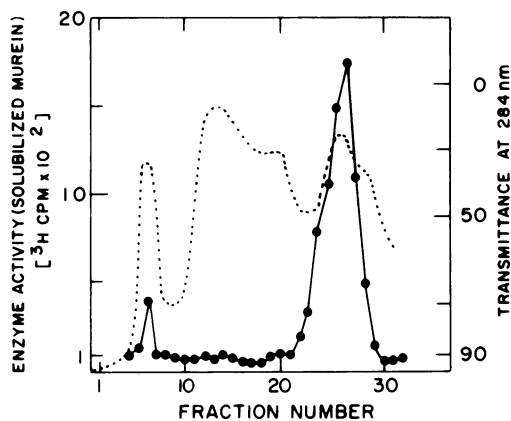

FIG. 1. Gel filtration of the lytic activity on a column (0.9 by 30 cm) of Bio-Gel A-1.5 m. Protein (2 mg), obtained from the crude E. coli W7 extract by ammonium sulfate precipitation (30 to 50% saturation), was dialyzed against 20% saturated $(NH_4)_2SO_4$ in 0.01 M sodium phosphate buffer (pH 6.9). Chromatography was in the same buffer containing 20% saturated $(NH_4)_2SO_4$. Fractions (0.5 ml) were collected at a flow rate of 37.5 ml/h. Enzyme activity of the fractions (5-μl aliquots) was assayed as described in Materials and Methods except that the incubation time was 1 h. Symbols: ····, transmittance at 284 nm; ●, enzyme activity.

increasing the molarity of the buffer to 0.4 M sodium acetate. The latter was, in fact, the ionic composition of the standard assay mixture used.

The dependence of the reaction on enzyme concentration and on incubation time is shown in Fig. 4A and B. There was a linear relationship between the rate of reaction and the incubation time (up to 15 min at 37 C with 0.4 μg of purified enzyme per ml). The rate of reaction increased linearly with the amount of
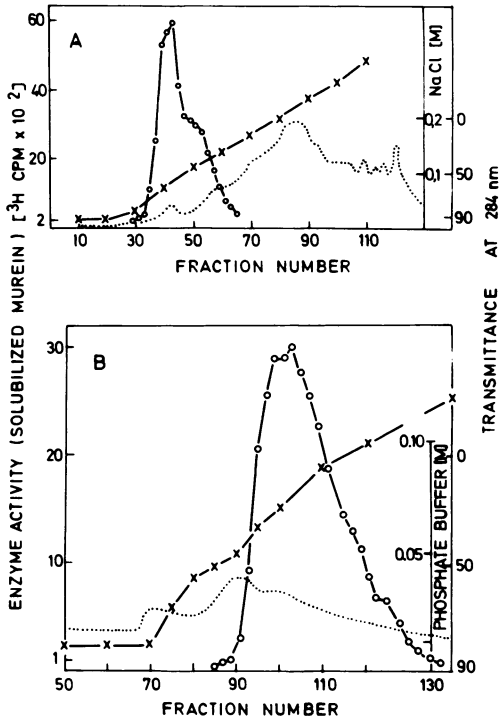
FIG. 2. *Elution profiles of enzyme from a column of (A) DEAE-cellulose DE 32 and of (B) Bio-Gel HTP hydroxylapatite (steps iv and v, respectively; see text). Symbols: ····, transmittance at 284 nm; O, enzyme activity; ×, molarity of eluant.*

substrate up to a concentration of 25 μg/ml (Fig. 4C).

The enzyme reaction follows normal Michaelis-Menten kinetics, as shown in the Lineweaver-Burk plot in Fig. 4D. The concentration of the substrate, which is a polymer, is given in milligrams per liter; the apparent K_m was found to be equal to 200 mg of murein sacculi per liter.

The appearance of low-molecular-weight reaction products during digestion of the murein by the purified enzyme was followed by gel filtration of incubation mixtures. Labeled sacculi were digested with purified enzyme for different times before fractionation on the gel column. Figure 5A shows a representative elution profile. The ultimate degradation products, muropeptides X and X', appear already after a short incubation time (10 min). Prolonged incubation (12 h) leads to almost a complete degradation of sacculi into low-molecular-weight components (approximately 80%). For further characterization of the reaction products, the fractions from the agarose column (Fig. 5) were lyophilized, desalted on Bio-Gel P-2, and fractionated on paper in solvent I (Fig. 5 B). The

main reaction products X/X' (Fig. 5, peak 3) run as a pair faster than C5/C6, the lysozyme digestion products of the sacculi (17), which were used as markers. The material from peak 2 also runs as a pair of compounds with R_f values between that of C5/C6 and C3/C4. The latter compounds are dimers of the muropeptides C5/C6, cross-linked at their peptide side chains (17). This suggests that the compounds found in peak 2 may be dimers of the material found in peak 3. The material from peak 1 (Fig. 5) moves slowly and appears to contain mainly oligomuropeptides.

Characterization of the digestion products X and X'. Compounds X and X' were isolated on a preparative scale as described above. X and X' migrated as single spots on paper electrophoresis, both at pH 3.5 and 6.5, with mobilities identical to those of C5 and C6, respectively. Both X and X' reacted with ninhydrin, and each had a free ε-amino group on the diaminopimelic acid as evidenced by dinitrophenylation studies. Analysis of purified X and
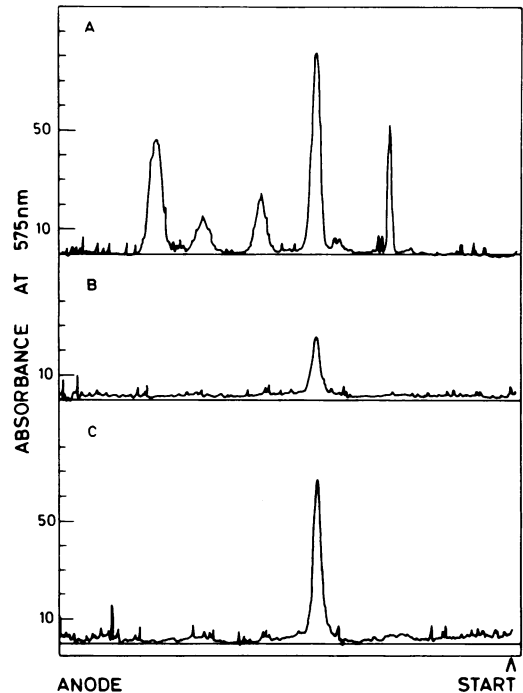

FIG. 3. *Electrophoresis of purified enzyme on sodium dodecyl sulfate-polyacrylamide gel. After the staining procedure with Coomassie blue, the optical density profile was measured with a Vitatron densitometer. (A) Enzyme (2 ug; 7,600 U/mg) was mixed with 2 μg of each standard protein (from left to right: myoglobin, chymotrypsin, ovalbumin, bovine serum albumin, β-galactosidase); (B) 2 μg of enzyme; (C) 4 μg of enzyme.*

X' showed that their chemical composition is identical to that of muropeptides C5 and C6, respectively (Table 3).

For studies of the structure of the disaccharide moiety of compounds X and X', the original mixture of the two compounds (X/X'), as obtained before fractionation by paper chromatography, was employed. X/X' had no reducing

TABLE 2. *Effect of cations on the activity of purified transglycosylase*[a]

Cations (as chlorides)	Concn of cation added (M)	Enzyme activity (reaction products; counts/min)
Na+	10^{-3}	370
	10^{-2}	400
	5×10^{-2}	1,100
	10^{-1}	3,470
K+	10^{-3}	450
	10^{-2}	450
Mg^{2+}	10^{-3}	875
	10^{-2}	2,245
Ca^{2+}	10^{-3}	990
	10^{-2}	3,050
Mn^{2+}	10^{-3}	685
	10^{-2}	1,190
Zn^{2+}SO$_4^{2-}$	10^{-3}	755
	10^{-2}	2,730

[a] Enzyme activity was assayed with [^{3}H]diaminopimelic acid-labeled sacculi (5×10^4 counts/min) as a substrate. The assays were complemented with the salts at the final concentrations listed above.
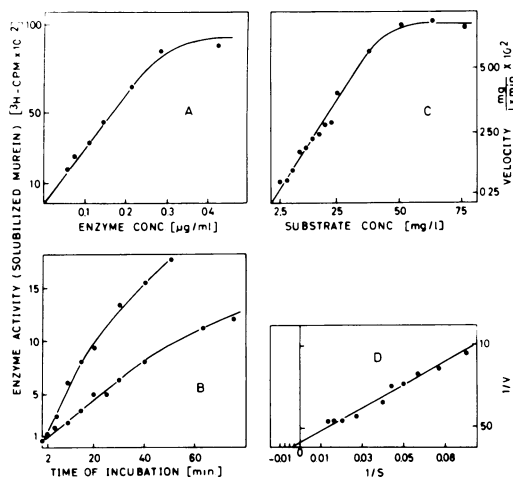

FIG. 4. *Characterization of the enzymatic properties of the purified transglycosylase.* (A) *Dependence of the rate of reaction on enzyme concentration. Standard assay conditions were used with the indicated concentrations of purified enzyme (4,000 U/mg).* (B) *Time course of reaction. The incubation mixture contained in standard assay buffer (in a final volume of 200 μl): 25 μl of ^{3}H-labeled sacculi (0.5 mg/ml; 1.5 × 10^6 counts/min per ml) and 0.14 μg of purified enzyme per ml (7,600 U/mg). Reaction was at 37 C (upper curve) and at 25 C (lower curve).* (C) *Dependence of reaction velocity on substrate concentration. Standard assay mixtures containing 0.04 μg of purified enzyme (7,600 U/mg) were incubated at 25 C with the indicated substrate concentrations.* (D) *Lineweaver-Burk plot of the data shown in panel C. S, Sacculi (milligrams per liter); V, rate of reaction measured as milligrams of substrate (^{3}H-labeled sacculi) solubilized/liter per minute.*
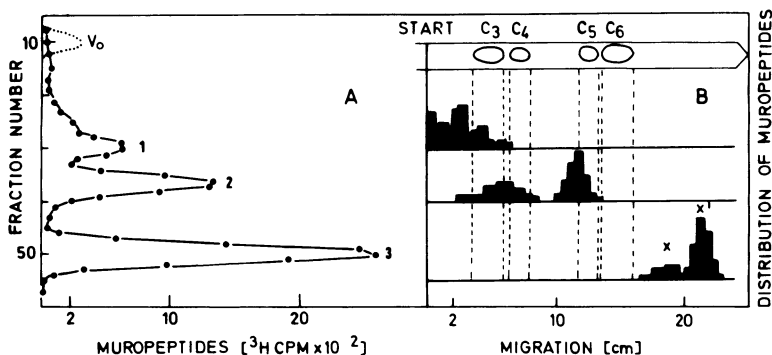

FIG. 5. *Separation of products released from murein sacculi by the action of the purified enzyme.* (A) *Elution profile of reaction products (obtained upon a 10-min incubation of sacculi with 0.25 μg of purified enzyme in 0.2 ml of solution under standard assay conditions) from a column (2 by 46 cm) of Bio-Gel A-1.5 m. Chromatography was performed in 0.1% sodium dodecyl sulfate. Fractions of 0.75 ml were collected, and aliquots of 250 μl were assayed for radioactivity.* (B) *Paper chromatography in solvent I of pooled fractions 1, 2, and 3 eluted from a Bio-Gel A-1.5 m column as shown in panel A. Radioactive materials on paper were detected with a chromatogram scanner.*

TABLE 3. *Analysis of muropeptide pairs C5-C6 and X-X' and of their ^{14}C-peracetylated derivatives*[a]

Determination	Molar ratio of constituents				Total amt of constituents (μmol)		
	X	X'	Acetylated		Acetylated		Tetra-O-[^{14}C]acetyl-N-acetyl-D glucosamine
			X/X'	C5/C6	X/X'	C5/C6	
Constituent							
Glucosamine	0.9	0.9	1.04	1.06	0.39	0.46	2.2
Muramic acid	0.8	0.7	1.09	1.00	0.41	0.43	
Diaminopimelic acid	0.8	0.8	0.92	0.91	0.34	0.39	
Glutamic acid	1.00	1.00	1.00	1.00	0.37	0.43	
Alanine	1.0	1.8	1.28	1.31	0.48	0.57	
D-[^{14}C]acetyl groups found after acetylation			3.47[b]	5.55[b]	1.67	2.45	8.8
Total radioactivity in sample					310,000[c]	455,000[c]	1,630,000[c]

[a] The acetylated derivatives were obtained after elution from paper chromatograms, as indicated in the text. Where molar ratios are given, glutamic acid was taken as 1.00.
[b] Residues per molecule.
[c] Counts per minute.

group (less than 0.05 μmol/μmol of X/X'), did not contain phosphate (less than 2 nmol/μmol) or sulfate, and gave a negative reaction for O-acetyls. No radioactivity was incorporated when X/X' was treated with NaB[^{3}H], nor did their chromatographic mobility change upon such treatment. No change in the chromatographic mobility of X/X' was observed when the compounds were treated under conditions (4 M NH$_4$OH; 37 C, 6 h) which lead to beta elimination (25). Moreover, no color developed in the Morgan-Elson test (18), also indicating that the compounds do not possess a reducing end group. In all the above-mentioned reactions, a mixture of the well-characterized disaccharide-peptides C5/C6 (17) was used as a reference and gave the expected positive results.

Acetylation of compounds X/X' with radioactive acetic anhydride was used to estimate the number of free hydroxyl and amino groups per molecule as compared with those available on compounds C5/C6. Our results (Table 3) show that, whereas six groups can be acetylated in muropeptides C5/C6 (the hydroxyls on carbons 1 and 6 of the N-acetylmuramic acid and those on carbons 3, 4, and 6 of N-acetyl-D-glucosamine and the free 6-amino group of diaminopimelic acid), only about four are acetylated in compounds X/X' (Table 3). Similar results on the total number of O-acetyl residues in C5/C6 and in X/X' were obtained from the nuclear magnetic resonance spectra of the peracetylated compounds in deuterated methanol (D. Mirelman, unpublished data). The spectra of O-acetylated C5/C6 and of X/X' were very

similar except in the high-field region (around 2.0 ppm) in which the acetate protons resonate. In muropeptides X/X', integration of the peaks in this region of the spectrum showed the presence of three O-acetyl groups and 2.8 N-acetyl groups, whereas in compounds C5/C6 five O-acetyl groups and three N-acetyl groups were found.

Since the structure of the *E. coli* sacculus is known in considerable detail (20) and since the amino acid composition of X and X' is identical to that of C5 and C6, respectively, it is safe to assume that the structure of their peptide moieties is the same. This is also supported by the results of dinitrophenylation studies, in which mono-dinitrophenyl-diaminopimelic acid was identified both in X/X' and in C5/C6.

The mixture of X/X' was not digested further by the purified transglycosylase. Likewise, no digestion of muropeptides C3, C4, C5 and C6 by the enzyme was observed.

Incubation of compounds C5/C6 and X/X' with an amidase preparation from *Streptomyces* (3) afforded free peptides only from C5/C6, whereas only traces of free peptides were detected in digests of X/X'. On the other hand, digestion of both mixtures of compounds with a crude glycosidase preparation from *C. lampas* released free N-acetylglucosamine which was identified on paper chromatograms. After digestion of C5/C6 with the *C. lampas* enzyme followed by reduction of the digest with NaB[^{3}H$_4$] and acid hydrolysis, both [^{3}H]glucosaminitol and [^{3}H]muramicitol were identified on the amino acid analyzer (14). With X/X', how-

ever, the only radioactive component detected after reduction and hydrolysis was [³H]glucosaminitol.

The N-acetylmuramyl-peptide obtained after digestion of compound X with the C. lampas glycosidase differed in its chromatographic mobility from the derivative obtained after digestion of compound C5 with the same enzyme. The digestion product from C5 had an R_{C_6} of 1.3 and the residue from X had an R_{C_6} of 1.6 (solvent I).

Under mild hydrolytic conditions (0.1 M HCl, 100 C, 15 min; 0.1 M NaOH, 37 C, 1 h), no appearance of reducing groups in compounds X/X' could be observed. Under stronger hydrolytic conditions (1 M HCl, 1 h at 100 C; 1 M H_2SO_4, 1 h at 100 C), approximately 50% of the reducing power (15) of muramic acid was unmasked (based on conversion to [³H]-muramicitol, see above). However, under these conditions similar amounts of free glucosamine, as well as peptides and free amino acids, were detected by paper chromatographic analysis of the reaction products, which indicated that the treatments had caused a partial breakdown of the compounds.

Most of the properties of X/X' can be accounted for by the structure proposed earlier by us (7, 9) in which the linkage between N-acetylglucosamine and N-acetylmuramic acid was 1 → 1, instead of the usual 1 → 4 bond found in all known muropeptides (20). However, the release of N-acetylglucosamine from compounds X/X' by the C. lampas glycosidase did not unmask the reducing group of the N-acetylmuramic acid moiety. This rules out the original suggestion of a 1 → 1 linkage in these compounds. The fact that the fully acetylated X/X' contains two acetyl groups less than the acetylated C5/C6 indicates that in the muramic acid moiety of X/X' the hydroxyls on carbon 1 and carbon 6 must be blocked and thus cannot be substituted by acetyl groups. Since no non-murein constituents were found in X and X', the most likely explanation for the absence of free hydroxyl groups on the muramic acid residue is that this residue possesses an internal (anhydro) structure, apparently 1 → 6 linked (Fig. 6).

A compound designated CA, identical to muropeptide X' both in chemical composition and chromatographic behavior, has recently been isolated as a reaction product from E. coli sacculi, digested by an enzyme present in phage lysates from E. coli (24). Mass spectroscopy of compound CA had indicated that the muramic acid is in the 1 → 6 anhydro form (24). Further evidence on the identity of CA and X' is based
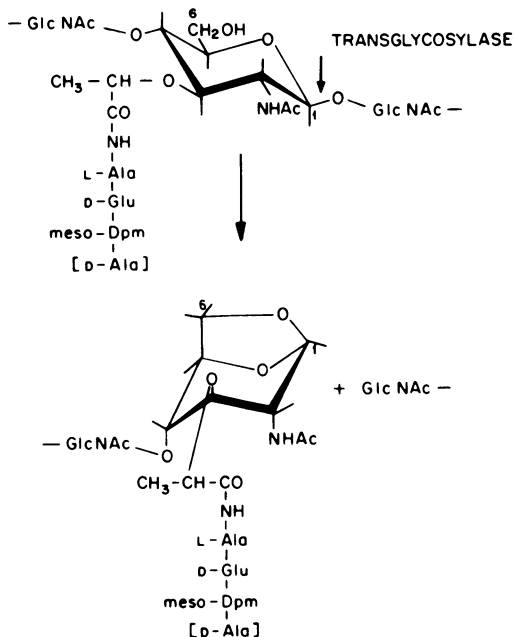

FIG. 6. Formation and structure of muropeptides X/X'. The scheme shows the conversion of the muramic acid moiety from the ⁴C₁ into the ¹C₄ conformation upon transglycosylase action. Muropeptide X lacks the terminal D-alanine present in X'. Dpm, Diaminopimelic acid; GlcNAc, N-acetyglucosamine.

on the finding that a sample of CA, kindly given to us by Alina Taylor, migrated on paper in solvent I at the same rate as X'. Because our transglycosylase is easily solubilized and should indeed appear in a phage lysate of E. coli cells, we believe that CA and X/X' are both produced by one and the same bacterial enzyme. This contrasts with the assumption that CA is product of a phage-coded enzyme (24).

Possible biological role of the novel transglycosylase. Studies with impure preparations of the transglycosylase had indicated that the enzyme might be one of the targets for penicillin since it was partially inhibited by high concentrations of the drug (7). It was hoped that extensive purification of the transglycosylase would shed more light on the biological significance of the penicillin effect. Since even the purified enzyme was only poorly inhibited by penicillin (150 μg/ml inhibited the enzyme by 20%), there is still doubt whether the transglycosylase is a target for penicillin action.

The transglycosylase described here converts the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine into an internal 1 → 6 anhydro N-acetylmuramyl (hemiacetal)

bond. The reaction performed by the enzyme is an intramolecular transglycosylation reaction. In analogy to the nomenclature for the glycogen-branching enzyme, our enzyme should be designated as murein: murein-6-muramyl transferase (internal cyclyzing). The formation of the anhydro bond by the enzyme may be considered as means for a conservation of the original energy present in the glycosidic bond.

The following alternative biological roles for the enzyme seem plausible. (i) The morphological changes of a bacterial cell during growth and division involve rearrangements within the sacculus molecule. There are indications that large sheets of murein are shuffled within the sacculus of a growing cell (19) and transglycosylation by enzymes of the lysozyme type could be responsible for the local rearrangement of the murein. In this case, however, a new glycosidic bond could be formed only at specific locations where enzymatic hydrolysis had occurred as the initial step preceding transglycosylation. Murein strands having an anhydro structure as an end group could be linked to the end groups of polysaccharide chains at any location in the sacculus without prior activation by hydrolysis of a glycosidic bond. Therefore, the novel transglycosylase could play a key role in the randomization of murein.

(ii) The enzyme could terminate murein strands at their potential reducing end. Nonreducing end groups have been detected in small amounts in intact murein sacculi from *E. coli* (17). The formation of the anhydro group at certain locations in the murein sacculi could perhaps also function as signals or controls for other enzymes involved in murein biosynthesis.

(iii) The enzyme may act differently in vivo and in vitro. The purified transglycosylase shows properties of a membrane-bound enzyme since it requires the presence of either Triton X-100 or serum albumin for its activity. In vivo, the enzyme could perhaps function in the direct transfer of murein-6-muramyl residues from one glycan chain to another, whereas the in vitro reaction products could be artifacts due to a misdirected intramolecular transfer reaction. Such side reactions could also happen to a minor extent in vivo and this could explain the occurrence of the nonreducing compounds found in lysozyme digests of sacculi (17).

There is a hope that detailed knowledge on the biochemical properties of the enzyme will enable the isolation of defective mutants in which the role of the enzyme can be studied and which would give further insight on the mechanism of murein biosynthesis in the growing cell and on the process of murein rearrangement within the sacculus.

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