The Mechanism of Soluble Peptidoglycan Hydrolysis by an Autolytic Muramidase

A PROCESSIVE EXODISACCHARIDASE*

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The action of purified N-acetylmuramoylhydrolase (muramidase, EC 3.2.1.17) of Streptococcus faecium ATCC 9790 on linear, uncleaved, soluble, peptidoglycan chains produced by the same organism in the presence of benzylpenicillin was characterized as a processive exodisaccharidase. Specific labels, one ([14C]Gal) added to the nonreducing ends of chains, and the other (H from [3H]NaBH₄) incorporated into the reducing ends of the chains, were used to establish that an enzyme molecule binds at the nonreducing terminus and sequentially hydrolyzes the glycosidic bonds, releasing disaccharide-peptide units. An enzyme molecule remains bound to a chain, and is not released at a detectable rate, until hydrolysis of that chain is complete. Reaction rates increased with the length of the polymer chain to give a maximum of 91 bonds cleaved/min/enzyme molecule for hydrolysis of a continuous polymeric substrate. The relationship between hydrolytic rate and glycan chain length is consistent with hydrolysis of bonds within the chain followed by slow release of enzyme from the distal, reducing terminus. This mechanism was experimentally confirmed by analysis of product formation during hydrolysis with stoichiometric mixtures of enzyme and soluble peptidoglycan chains. Kinetic analyses showed an apparent \( K_m \) of 0.17 \( \mu \)M for the enzyme, independent of substrate polymer length. The dissociation constant for the initial enzyme-substrate complex was calculated to be 1.5 \( nm \). Kinetic analyses are consistent with one catalytic site per enzyme molecule. The \( K_{cat}/K_m \) value of \( 9 \times 10^6 \) \( M^{-1} \) \( s^{-1} \) is near the limit imposed by diffusion for the initial hydrolytic events when long chains are hydrolyzed. The kinetic and physical properties of this muramidase are highly consistent with its location outside of the cellular permeability barrier and its ability to remain with and hydrolyze appropriate bonds in the cell wall in such an environment.

Autolytic peptidoglycan hydrolases (autolysins) are endogenous bacterial enzymes that hydrolyze specific bonds within insoluble cell walls, thereby causing the walls to lose their structural integrity and the cells to lyse unless external osmotic protection is provided (1). There have been numerous studies of the substrate specificities of such enzymes (see Ref. 2 for a recent review), and enzymes that hydrolyze the peptide, amide, and glycosidic bonds in wall peptidoglycans have been described (1-3). Several bacterial species have been found to contain enzymes that hydrolyze more than one type of bond within its peptidoglycan. However, there have been few studies of the mechanism by which bacterial autolysins attack their substrates. Such studies (4-7) have been limited by difficulties in obtaining pure enzymes and by insolubility of the chemically complex wall substrate. Most studies of the peptidoglycan hydrolases have used either (i) insoluble substrates, such as walls themselves, or insoluble peptidoglycan residues (obtained by treatment of walls with strong acids or bases); (ii) low-molecular-weight peptidoglycan fragments consisting of structural units of the wall peptidoglycan, such as DSP, or peptide cross-linked dimers of DSP; units obtained from enzymatic hydrolysates of walls, or (iii) low-molecular-weight oligosaccharides or peptides obtained via chemical synthesis or chemical hydrolysis of analogous substrates, such as chitin. The latter type of substrates have been very successfully used for the detailed examination of the mechanism of action of HEWL.

Recently it has become possible to isolate and purify s-peptidoglycans produced and secreted by penicillin-treated bacteria (8-12). These soluble polymers contain the bonds normally hydrolyzed by their respective autolysins in the same (or a very similar) relationship to nearby substituents as the homologous, insoluble cell wall. Thus, they can be regarded as "natural" substrates for autolysins, except for the absence of the peptide cross-bridges normally hydrolyzed by the \( \beta \)-lactam-sensitive peptidoglycan hydrolases.

As described elsewhere (13), high-molecular-weight s-peptidoglycans, produced by Streptococcus faecium ATCC 9790 in the presence of penicillin, have been isolated, purified, and chemically characterized. These polymers were shown to contain glycan strands of approximately uniform length that are fully substituted with peptides and thus consist of linear chains of DSP units. These chains are hydrolyzed by the

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* The abbreviations used are: DSP₁, DSP₂, DSP₃, DSP₄, disaccharide-peptide, glycan dimer of the disaccharide-peptide (MurNAc-J1-4-GalNAc-L-Ala-D-Gln-L-Lys-D-Ala-D-Ala) structural unit of the

** D-isoAsn cell wall peptidoglycan of S. faecium, etc., respectively; HEWL, hen egg-white lysozyme; s-peptidoglycan, soluble, linear, un-cross-linked peptidoglycan; SF muramidase(s), the N-acetylmuramoylhydrolase of S. faecium (or of the mutant strains) prepared as described (14, 15); Mur, muramic acid.
concanavalin A-Sepharose 4B-purified N-acetylmuramoylhydrodrolase (muramidase, EC 3.2.1.17) of *S. faecium* (14), producing DSP, as virtually its only product (15). Here, using a variety of techniques, including selective radiolabeling of the reducing and nonreducing ends of the chains, we demonstrate that this enzyme is a processive, exodisaccharidase that commences hydrolysis from the nonreducing ends of the s-peptidoglycan chains. The kinetic and rate constants have been defined for the initial interaction of enzyme and s-peptidoglycan, for the hydrolysis of internal glycosidic bonds, and for the release of the enzyme from the limiting s-peptidoglycan.

**MATERIALS AND METHODS**

**Experimental Procedures**—The organisms used, the preparation and characterization of s-peptidoglycans, SF muramidase, gel filtration, paper chromatography, determination of radioactivity, and chemicals and reagents have been described (13-15). The s-peptidoglycan chains are secreted into the medium by benzylpenicillin-inhibited cells (13). Unless otherwise indicated, the subculture for SF muramidase was s-peptidoglycan produced by Lpt-14 (a lytA-deficient strain of *S. faecium*) (16).

**Materials**—Galactosyltransferase (UDP-Gal-Glc or UDP-Gal-Gal, 4-6-D-galactosyltransferase, EC 2.4.1.22) from bovine milk (5 units/mg of protein) was purchased from Sigma, and uridine diphosphate-D-[U-14C]Gal, 305 mCi/mmol, from Amersham. Vancomycin was a generous gift of Eli Lilly. All other chemicals were as described (13-15).

**Preparation of Standard Markers for Paper Chromatography**—Preparative amounts of DSP, units (monomers) and the glycan-linked dimer, DSP2, (dimer) were obtained from hydrolysis of Lyt-14 s-peptidoglycan prepared from cells exposed to 50 μg/ml of benzylpenicillin (14). The labeled s-peptidoglycan (1 mg) was hydrolyzed by HEWL (20 units/ml) in 0.5 ml of 0.5 mM Na acetate, pH 6.0, for 30 min. In some experiments, radiolabeled s-peptidoglycan was hydrolyzed to produce labeled DSP, and DSP2. After desalting hydrolysates over Bio-Gel P-2 (Bio-Rad Laboratories, 38 × 125 cm), standards were separated by descending paper chromatography in Solvent I (butanolacetic acid-water, 4:1:5, v/v/v, upper phase) on Whatman 1 paper, for 72 h, and identified by comparison with known standards.

Unlabeled DSP1 and DSP2 were used to prepare [14C]Gal → DSP1 (or DSP2) and DSP1-[1-3H]muramiditol (or DSP2-[1-3H]Muramiditol) as described below. Excessively labeled alcohol derivatives of muramic acid and glucosamine were prepared for use as chromatography standards (13). Muramic acid and glucosamine were separated by ascending chromatography in Solvent II (butanolacetic acid-water, 3:1:1, v/v/v) on Whatman 1 for 4 h.

**Ligation of s-Peptidoglycan with [14C]Gal**—Galactosyltransferase has been shown to be capable of transferring [14C]Gal from UDP-[14C]Gal to the nonreducing terminus of GlcN-acid (17). Depending on the extent of labeling desired, from 20 to 200 μg of s-peptidoglycan in 40 μl of water was added to a reaction mixture containing 7 nmol of UDP-[14C]Gal (300 μCi/μmol), 0.06 units of galactosyltransferase (1 unit is defined as transferring 1.0 pmol of Gal from UDP-Gal to D-Glc per minute), 15 μmol of 3-(N-morpholino)propanesulfonic acid, 13 mM MnCl2, in a final volume of 230 μl. After incubation for 2 h at 37 °C, the s-peptidoglycan was separated from unincorporated UDP-[14C]Gal by gel filtration over a Sephadex G-100 column. Fractions containing [14C]Gal → s-peptidoglycan were pooled, lyophilized, and dissolved in 2.0 ml of 0.02% NaN3, pH 7.2 (adjusted with 0.01 M NH4OH). Galactosyltransferase was removed by affinity chromatography of the s-peptidoglycan on a vancomycin-Sepharose 4B column (18).

**Preparation of s-Peptidoglycans of Various Molecular Sizes**—s-Peptidoglycan was subject to partial enzymatic hydrolysis by HEWL (10 mg of Lyt-14 s-peptidoglycan, 0.1 mg of HEWL in 0.1 ml of 0.5 M Na acetate, pH 6.0, at 37 °C for 5 min). The reaction was stopped by heating (100 °C, 12 min) and the hydrolysate was fractionated on Sephadex G-100 into five fractions on the basis of reduced sugars elution profile. Each fraction was rechromatographed on Ultrogel AcA 34, and each of the peaks, eluted with water, was pooled, lyophilized, and stored at −70 °C. The average glycan chain length of each preparation was determined by the ratio of reducing groups before and after acid hydrolysis (19).

**Hydrolysis of s-Peptidoglycan by SF Muramidase**—s-Peptidoglycans of various molecular sizes (8-46 DSP, units) were incubated with SF muramidase (in various amounts from 10 to 1000 units) using the optimal conditions described (15). At intervals, samples were taken and monitored for reducing groups (19).

**Establishing the Concentration of SF Muramidase**—Purified SF muramidase, which was over 90% homogeneous as determined by sodium dodecyl sulfate-gel electrophoresis, was dialyzed against 2 mM K phosphate, pH 6.8. Equal volumes of dialysate and enzyme were dried to constant weight at 110 °C and weighed on a Cahn analytical balance. Samples of the same solution were used to establish standard curves of protein and enzyme activity. Experiments which depend on absolute enzyme concentration use these values for quantity of enzyme.

**RESULTS**

**Separation and Identification of Products of Enzymatic Hydrolysis**—The products of SF muramidase hydrolysis of s-peptidoglycan containing 14C from [14C]Glc in the glycan portion of the s-peptidoglycan and 3H from [3H]lys in the peptide side-chain portion of the s-peptidoglycan were analyzed by paper chromatography in Solvent I. Discrete and complete separation of the N-acetylglucosamine-N-acetyl muramic acid-peptide (DSP1) and glycan-linked dimer (DSP2) from oligosaccharide-peptides (DSP3) which remained at or near the origin was obtained. The chromatographic separation and structure of the products are shown in Fig. 1.

**Products of s-Peptidoglycan Hydrolysis**—The variety of

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A. R. Zeiger, personal communication.
products of HEWL action on *Micrococcus luteus* s-peptidoglycan resolved in Solvent I (9, 15) include the disaccharide GlcNAc-MurNAc and the glycan disaccharide dimer GlcNAc-MurNAc-GlcNAc-MurNAc, DSP, and DSP2, in addition to larger oligosaccharides and oligosaccharide-peptides (DSPn). Heterogeneity of products is due to the nature of the *M. luteus* s-peptidoglycan, which is only 40–50% substituted with peptides on MurNAc residues, presumably due in part to the action of the penicillin-insensitive amidase of *M. luteus* (9, 20). Because all of the MurNAc residues of a s-peptidoglycan of *S. faecium* are substituted with peptides, HEWL hydrolysis produced only two detectable low-molecular-weight products, DSP, and DSP2, plus longer oligosaccharide-peptides near the origin (15). In contrast to HEWL action, SF muramidase hydrolysis of Lyt-14 s-peptidoglycan resulted in the production of a large amount of DSP, and very little DSP2 (15). Radiolabeled s-peptidoglycan was prepared from the two autolysis-defective strains of *S. faecium* (Lyt-14 and Aut-3 (13)) to contain 14C (from [14C]Glc) in the glycan portion and 3H (from [3H]Lys) in the peptide portion. Complete hydrolysis of these polymers by the SF muramidase or partial hydrolysis by HEWL resulted in products that contained both 3H and 14C and had the same paper chromatographic mobilities as DSP1 and DSP2 (15). No evidence was obtained for the production of disaccharides or tetrasaccharides, indicating that, within the limits of detection, all MurNAc residues of the Lyt-14 and Aut-3 s-peptidoglycan were substituted with peptides.

**Kinetics of Hydrolysis of s-Peptidoglycan Chains of Different Chain Lengths by the SF Muramidase**—Lineweaver-Burk plots of hydrolysis of s-peptidoglycan chains of 13 ± 1, 22 ± 2, 28 ± 2, and 46 ± 2 DSP units by the SF muramidase showed decreased maximum velocities and decreased apparent K values with decreasing chain length when substrate concentration was expressed in terms of DSP units (Fig. 2, inset). However, plots of the same data in which substrate concentration is expressed in terms of s-peptidoglycan chains (irrespective of chain length), resulted in similar K values (average K = 0.175 ± 0.03 μM; Fig. 2). In contrast, Vmax was strongly dependent on the degree of polymerization size of the substrate, with turnover numbers ranging from 7 to 19 mol of DSP1 released min⁻¹ mol⁻¹ of enzyme (Fig. 2). To determine the true K values, the substrate concentration was expressed in terms of free substrate concentration, while taking into account any depletion of free substrate by the enzyme-substrate complex (21). Lineweaver-Burk plots of the hydrolyses of s-peptidoglycans by the SF muramidase were used to generate apparent K values. By using data generated from the hydrolyses of s-peptidoglycans of various lengths with various concentrations of enzyme, the relationship of K to enzyme concentration and to chain length can be established. Fig. 3A shows a plot of apparent K values (in micromolar s-peptidoglycan chains) versus enzyme concentration (in units of activity and enzyme molarity). The intercept on the ordinate gives an approximation of the true K value, where substrate depletion by the enzyme does not occur. This K value of 0.17 μM indicates a relatively high affinity of the SF muramidase for the substrate and is independent of glycan chain length (Figs. 2 and 3).

**Processive Action of SF Muramidase**—The parallel release of DSP1 units and reducing groups (15) and the detection of DSP as the major product of hydrolysis (15) are consistent with an exohydrolytic mode of catalysis. The lack of higher-
molecular-weight products during hydrolysis by SF muramidase (Fig. 2) clearly distinguishes the SF muramidase from HEWL, which has been characterized as an endoglycosidase (22-25). To determine whether the SF muramidase is processive (catalyzing multiple hydrolytic events before enzyme release), several experiments were conducted, including (i) competition experiments in which substrate was added to enzyme which was prebound to substrate under conditions where the catalytic rate is insignificant (0 °C); and (ii) determination of the initial hydrolytic site of the SF muramidase on the s-peptidoglycan by the use of end-labeled polymers.

In the experiment shown in Fig. 4 (bottom panel), latent (zymogen form) SF muramidase was prebound to excess [3H] s-peptidoglycan. Following proteinase activation of the latent enzyme, an equal amount of [14C] s-peptidoglycan was added, followed by hydrolysis at a permissive temperature. Samples were taken at intervals, the reactions were stopped (100 °C, 7 min), and samples were analyzed by paper chromatography in Solvent I. Preincubation with [3H] s-peptidoglycan resulted in the production of only [3H]DSP units, for the first 2 min of incubation (Fig. 4, bottom). In contrast, release of [14C] DSP was observed only after a delay of approximately 2 min, after which release of [3H]DSP, and [14C]DSP, paralleled each other. This initial release of product from prebound substrate clearly demonstrates preferential hydrolysis of prebound substrates and indicates that free DSP, a s-peptidoglycan does not readily exchange with existing enzyme s-peptidoglycan complexes.

As a control for these competition experiments, a known endoglycosidase, HEWL, was tested in a similar experiment using the same substrates. The experimental design was identical to that for the SF muramidase, but with the modification of assaying for both DSP and DSPs, products, since the random pattern of hydrolysis by HEWL results primarily in these products (9, 21-24). In contrast to the SF muramidase, HEWL hydrolysis did not show a preferential release of 3H-labeled products from the substrate to which enzyme was prebound (Fig. 4, upper panel) and instead resulted in the parallel release of both 3H- and 14C-labeled products throughout the incubation.

Several variations on the type of the experiments described in Fig. 4 were performed. For example: (i) latent SF muramidase was first activated with bovine plasma albumin (containing activating proteinase) before prebinding to [3H] s-peptidoglycan at 0 °C; (ii) latent SF muramidase was prebound to [14C] s-peptidoglycan at 0 °C before activation and exposure to [3H] s-peptidoglycan. The results of both of these experiments were quantitatively indistinguishable from those shown in the lower panel of Fig. 4.

In summary, all experiments demonstrated that the SF muramidase preferentially hydrolyzed the glycans chains s-peptidoglycan to which it was prebound. Furthermore, the SF muramidase maintained its initial enzyme-substrate complex for approximately 2 min (Fig. 4). To examine further the sequential nature of hydrolysis, preactivated SF muramidase was prebound to excess [14C] s-peptidoglycan at 0 °C before activation and exposure to [3H] s-peptidoglycan. The reaction mixtures were rapidly brought to 37 °C. Release of [14C]DSP units occurred at equal rates independent of the presence of varying amounts of competing, unlabeled substrate. The independent rate of [14C]DSP formation continued for about 2 min. After 2 min, the rate of release of [14C] DSP units depended on the concentration of unlabeled s-peptidoglycan added. As the concentration of the unlabeled
Processive Hydrolysis of Soluble Peptidoglycan

Fig. 4. Demonstration of the high affinity of the SF muramidase for s-peptidoglycan. The preferential hydrolysis of prebound chains by the SF muramidase (bottom) and random hydrolysis of bound and unbound chains by HEWL (top) are shown. In the experiment shown in the bottom panel, SF muramidase and $[^3H]$Glu-s-peptidoglycan were incubated in 1.0 ml of 10 mM Na phosphate, pH 6.8, at 0 °C for 5 min. Bovine plasma albumin (75 μg/ml) was then added, and, after 3 min at 0 °C (to allow proteinase activation of the zymogen form), $[^14C]$Glu-s-peptidoglycan was added and the temperature was rapidly brought to 37 °C. The final concentrations of reactants in 1.2 ml of 10 mM Na phosphate, pH 6.8, were: 2.8 nM SF muramidase, 174 nM $[^3H]$s-peptidoglycan chains (DSP₄₋₅) and 174 nM $[^14C]$s-peptidoglycan chains. In the experiment shown in the upper panel, HEWL (5 units/ml) was added instead of the SF muramidase. A second control experiment consisted of SF muramidase being prebound to both $[^3H]$s-peptidoglycan and $[^14C]$s-peptidoglycan (bottom, solid line). At timed intervals, during incubation at 37 °C, samples were removed, the reaction stopped (100 °C for 7 min), and the products of hydrolysis identified by paper chromatography as described in Fig. 1. As shown in the top part of the figure, HEWL hydrolysis resulted in the parallel release of $[^3H]$DSP₁₋₂ (○) and $[^14C]$DSP₁₋₂ (○) products. A preference for hydrolysis of prebound $[^3H]$s-peptidoglycan was not seen. In contrast, action of the SF muramidase (bottom) resulted in the preferential release of $[^3H]$DSP, for the initial 2 min, after which both $[^3H]$DSP, and $[^14C]$DSP, were released at a parallel rate. The solid line in the bottom graph represents the parallel release on $[^3H]$- and $[^14C]$DSP, products when both $[^3H]$- and $[^14C]$s-peptidoglycan were bound to the SF muramidase in the control.

Site of Binding and Direction of Hydrolysis—The results described above suggested a single binding site for the enzyme on an s-peptidoglycan chain, most likely at the reducing or nonreducing end of a glycan chain. Therefore, s-peptidoglycan was radiolabeled with $^3$H at the reducing terminus by reduction of the terminal MurNAc residue with $[^3H]$NaBH₄. Similarly, s-peptidoglycan was radiolabeled with the addition of $[^14C]$Gal to the nonreducing terminus via the action of galactosyltransferase (17). The hydrolysis of doubly labeled s-peptidoglycan by the SF muramidase in a molar ratio of enzyme:substrate of approximately 5 resulted in the rapid and preferential production of $[^14C]$Gal → DSP₁ and DSP₂ (Fig. 6). The initial burst of $^3$C → DSP₁ and DSP₂ indicated that the SF muramidase preferentially hydrolyzes bonds in the s-peptidoglycan at the nonreducing terminus. A delayed burst of $^3$H-labeled products was observed, with a midpoint at 2...
the competition studies that indicated a period of approximately 2 min when SF muramidase, prebound to s-peptidoglycan, could not be displaced (Figs. 4 and 5). The release of \(^3\)H-labeled products at approximately 2 min is consistent with the apparent turnover number (\(-2l/min\)) obtained from the reducing end of the polymer is normally the reducing end.

substrate polymer and following the extent of DSP, release of \[^{14}\text{C}\]Gal-labeled, and \(^3\)H-labeled product is consistent with the predicted turnover number of 91 catalytic events/min at 37 °C for a continuous polymer of DSP units (see Fig. 3B). This result indicates that the observed dependence of \(V_{\text{max}}\) on s-peptidoglycan chain length (Figs. 2 and 3B) is due to the slow rate of enzyme release from the limit digest fragment and that the enzyme cleaves the DSP units at a uniform rate until approaching the limit digest fragment.

The results of Fig. 7 also provided the stoichiometry of enzyme catalytic sites. Following the first burst of product formation (after 1 min, Fig. 7), 0.12 nmol of s-peptidoglycan (DSP\(_{\text{u}}\)) was released by 0.14 nmol of enzyme. The enzyme thus catalyzed the hydrolysis of 0.86 mol of s-peptidoglycan/catalytic cycle/polypeptide chain.

**DISCUSSION**

Several muramidases, such as the well-studied HEWL, are known to randomly hydrolyze \(\beta-1,4\) linkages between MurNAc and GlcNAc (22–24) and, in the case of several lysozymes, to carry out transglycosylations (24). The data provided in this and previous studies (13, 15) demonstrate that the purified endogenous SF muramidase is a specific exodisaccharidase. Comparisons of the action of the SF muramidase with that of HEWL on s-peptidoglycans showed that, in contrast to HEWL, SF muramidase hydrolysis resulted in only one major product, DSP\(_1\), even after short incubations (Fig. 4; see also Ref. 15). The parallel production of DSP\(_1\) units and hydrolysis of glycosidic bonds, as indicated by the generation of new reducing groups (19), indicated that the SF muramidase is an exodisaccharidase. The maximum velocity of the SF muramidase varied with the chain length of the...
FIG. 6. Direction of hydrolysis by SF muramidase. The reaction mixture contained dual radiolabeled s-peptidoglycan (0.4 nmol of chains), labeled with 6 and 51% efficiency by [14C]Gal and 3H, respectively (as described under "Materials and Methods"), were mixed with 833 units of activated SF muramidase (0.56 nmol) and 10 mM Na phosphate, pH 6.8, in a total volume of 600 μl, and incubated at 0°C. This represents an enzyme:substrate ratio of 1.4:1. The reaction mixture was quickly brought to 37°C, and samples of 100 μl were taken at timed intervals. Following heat inactivation at 100°C for 5 min, the products were separated in Solvent I and quantified by detection of radioactivity. Total [14C]Gal + [3H] products (○) precede release of [3H]-labeled products (△) by 2 min. The inset demonstrates the fraction of total counts which appeared as [14C]Gal-DSPI (□), [14C]Gal-DSPI (□), DSP1 ([3H]muramicitol) (●), and DSP1 ([3H]muramicitol) (○).

FIG. 7. Time course of s-peptidoglycan hydrolysis. Latent SF muramidase (0.14 nmol) was preincubated at 0°C for 5 min with 0.8 nmol of [14C]glucose-DSPI and converted from the zymogen by 5 min of incubation at 0°C with 0.4 μg/ml of trypsin. The total reaction mixture (0.5 ml) was rapidly heated to 30°C, and, at the indicated intervals, samples (20 μl) were taken and boiled to terminate the reaction. The amount of DSP1 formed was established by chromatography and liquid scintillation counting as described under "Materials and Methods" and in the legend to Fig. 2.

substrate; however, the apparent Kₘ remained constant when substrate concentration was expressed in terms of s-peptidoglycan chains (Fig. 2). These observations clearly distinguish the actions of SF muramidase and HEWL. For example, HEWL hydrolysis of oligosaccharides greater than six saccharides (which is the minimum for maximum productive binding by HEWL (26)) shows little difference in kinetic constants as a function of polymer size (27). This relatively constant value of Kₘ in terms of substrate molarity but not in DSP1 units (Fig. 2) suggested a unique binding site on s-peptidoglycan chains. This site appears to be the nonreducing end of the polymer. There are several possible explanations for the observed difference in apparent Vₘₐₓ with different polymer sizes. These include: (i) that internal chain cleavages are slower on shorter chains; (ii) that there is a decreased cleavage rate as the terminal residue is approached; or (iii) that the initial cleavage is slow compared to the others.

To distinguish between initial binding at an internal residue or at a terminus, a series of competition experiments was carried out, in which the SF muramidase was prebound to labeled s-peptidoglycan under conditions where no significant catalysis occurs. Once bound to a glycan chain, the SF muramidase sequentially hydrolyzed the entire glycan chain to DSP1 before becoming available to combine with another glycan chain (Figs. 4, 5, and 7). Both the zymogen and active form of the SF muramidase bind tightly to the s-peptidoglycan, similar to the binding of SF muramidase to intact cell walls (28, 29). The time (~2 min) required to completely hydrolyze a glycan chain in competition experiments is consistent with the kinetically derived turnover number of the enzyme, suggesting that initial binding is at a terminus, with sequential hydrolysis of nearly the entire s-peptidoglycan...
chain occurring before dissociation of the enzyme-substrate complex. SF muramidase hydrolysis of peptidoglycan chains labeled extrinsically at the reducing terminus with $[^3H]$NaBH$_4$, and at the nonreducing terminus by the addition of $[^{14}C]$Gal, showed preferential release of $^{14}$C-labeled products from the nonreducing terminus, and a 2-min delay in release of $^3$H-labeled products from the reducing terminus (Fig. 6). In summary, the kinetic and labeling experiments indicated that the SF muramidase binds to the nonreducing terminus of the s-peptidoglycan followed by the sequential hydrolysis of sensitive bonds and the release of DSP$_2$. Enzyme is not released from the complex until hydrolysis is complete. Thus, it can be concluded that SF muramidase is a processive exodisaccharidase. The observation that the DSP$_2$ moiety is not a good substrate for the SF muramidase (having a 10- to 100-fold lower reactivity than the DSP$_{1n}$-length s-peptidoglycan substrate) and that DSP$_2$ is present in significant amounts following extensive hydrolysis of s-peptidoglycan indicates that the final DSP$_2$ unit is not usually cleaved. The long lag period which follows complete hydrolysis of a s-peptidoglycan chain indicates that hydrolysis is rapid but release of the final DSP$_2$ unit is slow, becoming a rate-limiting factor in the overall steady-state pattern when enzyme is present at a low molar ratio with respect to DSP$_n$. A provisional model for this action is shown in Fig. 8.

The calculation of rate constants for this mechanism and the determination of binding site stoichiometries requires the concentration of active (catalytic) sites in SF muramidase preparations. Active site titration with $[^3H]$Gal-labeled substrate and analysis of the initial burst of product at saturating substrate concentration (Fig. 7) provides the titration of catalytic sites per unit of enzyme activity. The molar ratio of product released in the initial burst to the number of active sites is approximately one catalytic site per molecule.

The inability of the SF muramidase to show transglycosylase activity (15) and the absence of product inhibition by the DSP$_1$, simplify the construction of a model to be provisionally assigned for the mechanism of action of the SF muramidase. Similar cases have been presented by Bailey and French (30).

A kinetic representation of the mechanism of action of the SF muramidase can be diagrammed, as shown in Scheme 1.

\[
E + DSP_1 \rightarrow DSP_1 + E \quad (k_4)
\]

\[
P_1 \rightarrow E + DSP_1 \quad (k_5)
\]

\[
P_2 \rightarrow E + DSP_2 \quad (k_6)
\]

\[
P_{n-2} \rightarrow E + DSP_{n-2} \quad (k_{12})
\]

\[
P_{n-1} \rightarrow E + DSP_{n-1} \quad (k_{13})
\]

\[
P_n + E \rightarrow E \cdot DSP_n \quad (k_1)
\]

\[
P_n + E \rightarrow E \cdot DSP_n \quad (k_2)
\]

\[
P_n + E \rightarrow E \cdot DSP_n \quad (k_3)
\]

where DSP$_n$ is the only form of the substrate to complex with $E$. The consequence of $k_1$, $k_2$, and $k_3$ being negligible compared to $k_4$, (no dissociation of the $E$-DSP$_n$ complex) results in the progression of the SF muramidase along the s-peptidoglycan chain, releasing DSP$_2$ (P). The last fragment to be released is usually DSP$_n$, since approximately 5% of the total radioactivity of labeled DSP$_n$ is found as DSP$_1$, following hydrolysis by SF muramidase (15). However, when the reducing end is converted to the muramicitol by borohydride reduction, a substantial fraction is released as the DSP$_1$, muramicitol (Fig. 6), suggesting that either DSP$_1$ or DSP$_2$ can be the terminal product of DSP$_n$, hydrolysis. The rate constants $k_1$, $k_2$, and $k_3$ can be used to define the $K_m$ value, the dissociation constant for the $E$-DSP$_2$, complex, and the rate at which internal chain DSP$_n$ bonds are hydrolyzed. The rate constant $k_4$ represents the release of the terminal DSP$_2$ (or DSP$_1$) unit to regenerate free enzyme. The maximum rate of catalysis by SF muramidase is influenced by the chain length of the s-peptidoglycan substrate (Fig. 2). Extrapolation of the rate to a continuous polymer gives a turnover number of 91 events min$^{-1}$, and this value is confirmed experimentally by the burst kinetics shown in Fig. 7. This value provides a reasonable estimate of $k_4$. The $K_{cat}/K_m$ value is $9 \times 10^5$ M$^{-1}$ s$^{-1}$ using the $K_m$ of 0.17 $\mu$M (Fig. 3). This value is near the diffusion-controlled limit of $\sim 5 \times 10^7$ M$^{-1}$ s$^{-1}$ for the second-order rate.

**Fig. 8.** Model for hydrolysis of s-peptidoglycan by the SF muramidase. Shown are the stages of initial binding (a), initial hydrolyses (b), continued sequential hydrolysis (c), and hydrolysis of the last few bonds and release of DSP$_2$ and enzyme, before binding to another s-peptidoglycan strand (d).

**Scheme 1**
constant describing the initial interaction of 86,000 (SF muramidase) and 50,000 $M_0$ (DSP$_1$) polymers and the cleavage of the first glycosidic bond (31). The size of the s-peptidoglycan substrate reduces the upper limit of $10^6$ to $10^8$ M$^{-1}$ s$^{-1}$ usually obtained for the second-order rate constant for interaction between small molecules and enzymes (32). The subsequent cleavages are not subject to diffusion control, since enzyme and substrate are in close proximity, and the release of enzyme from the substrate cannot be experimentally demonstrated (e.g., Figs. 4 and 6). The comparable constant has been reported to be $1.7 \times 10^4$ M$^{-1}$ s$^{-1}$ for HEWL using (GlcNAc-MurNAc)$_3$ as the ligand (22). These calculations indicate that the muramidase is approximately 500 times more efficient in forming product from long peptidoglycans than is HEWL. The evolution of the catalytic potential has maximized the turnover number while retaining sufficiently tight binding to prevent dissociation of the enzyme from its normal substrate of cell wall polymer.

The $K_m$ value for SF muramidase is constant as a function of chain length and can be used to calculate the dissociation constant for the enzyme-substrate complex during its action on an extended polymer. The $K_m$ is described by the constants $(k_0 + k_0)/k$ for the polymer and the dissociation constant, $K_D = k_0/k$. $K_m$ can thus be estimated to be $1.5 \times 10^4$ M$^{-1}$ for the reaction E + DSP, $= E$-DSP, where $n$, $p$, and $q$ are large. The first-order rate constant for dissociation, $k_0$, is approximately 0.013 s$^{-1}$, consistent with the results of Figs. 4 and 5 which demonstrate that the polymer does not readily dissociate once the Michaelis complex is formed. These constants are the limiting values for an s-peptidoglycan substrate which is continuous, DSP$_n$, where $n$ is large. The value of 91 min$^{-1}$ for $k_0$ in large DSP polymers may also be correct for hydrolysis of smaller s-peptidoglycans, as indicated by the linear plot of Fig. 3B. In this mechanism, hydrolysis of DSP$_n$ from the polymer always occurs at 91 min$^{-1}$ and release of the final DSP$_2$ is slow and constant for any starting s-peptidoglycan. Release of DSP$_2$ is shown by a distinct rate constant, $k_b$ in the reaction mechanism (Scheme 1), since hydrolysis of every peptidoglycan chain ends with the same E-DSP$_2$ complex. The value of $k_b$ can be estimated from Equation 1.

$$k_b = (n - 2)/V_{max} - (n - 2)/k_0$$

(1)

where $n$ is the length of the DSP$_n$ s-peptidoglycan and $V_{max}$ is the maximum rate at saturating concentrations of s-peptidoglycans of various $n$ values (Fig. 2). Equation 1 can be rearranged to predict that a double-reciprocal plot of apparent $V_{max}$ as a function of chain length should give a linear plot as shown in Equation 2.

$$\frac{1}{V_n} = k_b \frac{1+1/k_0}{n-2}$$

(2)

The slope of this line yields $k_b$, the average time for dissociation of the E-DSP$_2$ complex. Analysis of the data from Fig. 3B, according to Equation 2, gives a value for $k_b$ of 1.5 min; the average residence time of DSP$_2$ on SF muramidase under normal assay conditions ($37^\circ C$). The observed lag period of 2.5 min at $30^\circ$C (Fig. 7) is consistent with the calculated lag period of 1.5 min at $37^\circ$C from the results of Figs. 2 and 3. A mechanism where the rate of each hydrolytic event is uniformly dependent on chain length can be eliminated by the results of Fig. 7 which clearly demonstrates the lag period for release of DSP$_2$. The possibility that enzyme attaches to the nonreducing end (or elsewhere) and requires 1.5 min to begin hydrolysis at the nonreducing end is eliminated by results of Figs. 4-6, which demonstrate no lag in the initial phases of hydrolysis.

Although the SF muramidase has been known for over 15 years (33), its reaction kinetics were not previously characterized. The present studies establish that the enzyme recognizes the reducing end of peptidoglycan chains and does not initiate cleavage at internal glycan residues. The enzyme-substrate complex has a large commitment to catalysis, with the substrate release rate being nearly insignificant compared to the initiation of hydrolysis. These factors cause the enzyme to act continuously on a single s-peptidoglycan chain. Enzyme release does not occur until all glycan bonds have been cleaved, with the exception of the final DSP$_2$ unit. The results suggest relatively rapid hydrolysis of internal glycan residues followed by a pause as the last DSP units are released or hydrolyzed and released.

In vivo, this enzyme attaches tightly to the cell wall (28). The kinetic properties of high binding affinity ($\sim 10^{-9}$ M for the dissociation constant) and lack of release during catalysis are well suited to the enzyme location outside of the cell-permeability barrier and to its proposed role as an exoskeleton-remodeling enzyme (1). Although the turnover rate of the enzyme is relatively slow, any increase in rate would necessitate a decreased affinity, since the $V_{max}/K_m$ value is near diffusion limits. A lowered affinity would be unsuitable for a cell wall-remodeling enzyme, since it would risk loss of the enzyme from the cell wall to the medium.

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REFERENCES
1. Daneo-Moore, L., and Shockman, G. D. (1977) Cell Surf. Rev. 4, 597-713
2. Rogers, H. J. (1979) in Microbial Polysaccharides and Polysaccharides (Berkeley, R. C. W., Gooday, G. W., and Ellwood, D. C., eds), pp. 237-268, Academic Press, New York
3. Ghuysen, J.-M. (1968) Bacteriol. Rev. 32, 425-464
4. Herbold, D. R., and Glaser, L. (1975) J. Biol. Chem. 250, 7231-7238
5. Kawagishi, S., Araki, Y., and Ito, E. (1979) FEBS Lett. 29, 20-22
6. Kawagishi, S., Araki, Y., and Ito, E. (1980) Eur. J. Biochem. 112, 273-281
7. Bechara, E. H., Keck, W., de Pedro, M. A., and Schwarz, U. (1981) Eur. J. Biochem. 116, 355-358
8. Keglevic, D., Ladesic, B., Hadzija, O., Tomasich, J., Valinger, Z., Pokorny, M., and Naumski, R. (1974) Eur. J. Biochem. 42, 389-400
9. Morelman, D., Bracha, R., and Sharon, N. (1974) Biochemistry 13, 5043-5058
10. Tynecka, Z., and Ward, J. B. (1975) Biochem. J. 146, 253-267
11. Waxman, D. J., Yu, W., and Strominger, J. L. (1980) J. Biol. Chem. 255, 11577-11587
12. Zeiger, A. R., Wong, W., Chatterjee, A. N., Young, F. E., and Fussell, C. V. (1982) Infect. Immun. 37, 112-118
13. Barrett, J. F., and Shockman, G. D. (1984) J. Bacteriol. 159, 511-519
14. Kawamura, T., and Shockman, G. D. (1983) J. Biol. Chem. 258, 9514-9521
15. Barrett, J. F., Schramm, V. L., and Shockman, G. D. (1984) J. Bacteriol. 159, 520-526
16. Cornett, J. B., Redman, B. E., and Shockman, G. D. (1978) J. Bacteriol. 133, 531-540
17. Schindler, M., Morelman, D., and Schwarz, U. (1976) Eur. J. Biochem. 71, 131-134
18. de Pedro, M. A., and Schwarz, U. (1980) FEBS Microbiol. Lett. 9, 215-217
19. Park, J. T., and Johnson, M. J. (1949) J. Biol. Chem. 181, 149-151
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20. Jensen, S. E., and Campbell, J. N. (1976) J. Bacteriol. 127, 319-326
21. Cha, S. (1970) J. Biol. Chem. 245, 4814-4818
22. Chipman, D. M., Pollock, J. J., and Sharon, N. (1968) J. Biol. Chem. 243, 487-496
23. Chipman, D. M. (1971) Biochemistry 10, 1714-1722
24. Osserman, E. F., Canfield, R. E., and Beychok, S. (eds) (1974) Lysozyme, Academic Press, New York
25. Schinder, M., Mirelman, D., and Sharon, N. (1977) Biochim. Biophy. Acta 482, 386-392
26. Rupley, J. A. (1967) Proc. R. Soc. Lond. B Biol. Sci. 167, 416-428
27. Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1972) in The Enzymes (Boyer, P. D., ed) Vol. 7, pp. 665-868, Academic Press, New York
28. Shockman, G. D., and Cheney, M. C. (1969) J. Bacteriol. 98, 1199-1207
29. Pooley, H. M., and Shockman, G. D. (1969) J. Bacteriol. 100, 617-624
30. Bailey, J. M., and French, D. (1957) J. Biol. Chem. 226, 1-14
31. Hammes, G. G. (1978) Principles of Chemical Kinetics, Academic Press, New York
32. Hammes, G. G., and Schimmel, P. R. (1970) in The Enzyme (Boyer, P. D., ed) Vol. 2, pp. 87-114, Academic Press, New York
33. Shockman, G. D., Thompson, J. S., and Conover, M. J. (1969) Biochemistry 6, 1054-1065