Biosynthetic Elongation of Isolated Teichuronic Acid Polymers via Glucosyl- and N-Acetylmannosaminuronosyltransferases from Solubilized Cytoplasmic Membrane Fragments of Micrococcus luteus

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Teichuronic acid of Micrococcus luteus is a cell wall polysaccharide, covalently linked to peptidoglycan, which consists of alternating β-glucose and N-acetyl-β-mannosaminuronic acid (ManNAcA) residues (9). The disaccharide repeat unit has the structure →4)-β-D-ManNAcAp-(1→6)-α-D-Glcp-(1→ (2, 7). Teichuronic acid is readily liberated from cell walls by cleavage of the phosphodiester bond by which it is linked to peptidoglycan (1, 3). Analysis of purified teichuronic acid by polyacrylamide gel electrophoresis reveals a ladderlike banding pattern, each band of which differs from its two nearest-neighbor bands by one disaccharide repeat unit, as discussed in the accompanying paper (16).

Biosynthesis of teichuronic acid catalyzed by cytoplasmic membrane fragments of M. luteus requires uridine diphosphate β-glucose (UDP-glucose), uridine diphosphate N-acetyl-β-mannosaminuronic acid (UDP-ManNAcA), and uridine diphosphate N-acetyl-β-glucosamine (6, 8). Elongation of endogenous teichuronic acid by wall membrane preparations requires only UDP-glucose and UDP-ManNAcA (15). The transferases which catalyze incorporation of monosaccharide residues can be solubilized with Triton X-100 (13), but Thesit (dodecyl alcohol polyoxyethylene ether) is a more effective solubilizing agent and is compatible with subsequent purification of the glucosyltransferase (4).

We now report the utilization of detergent-solubilized enzymes from cytoplasmic membrane fragments to catalyze the elongation of exogenous teichuronic acid. Analysis of the biosynthetic product by polyacrylamide gel electrophoresis demonstrates conclusively the addition of a single glucosyl residue when UDP-glucose is the only sugar nucleotide present in incubation mixtures and the addition of multiple disaccharide repeat units when both UDP-glucose and UDP-ManNAcA are present.

MATERIALS AND METHODS

Materials. UDP-[14C]glucose (250 mCi/mmol) was prepared from [14C]glucose and UDP with a crude yeast extract (10, 14). UDP-ManNAcA was prepared from UDP-GlcNAc by using a crude extract of Escherichia coli O14:K7:H− (5, 10).

Soluble enzymes. Glycosyltransferase and N-acetylmannosaminuronosyltransferase were solubilized by methods previously described (4). Briefly, a 3% solution of Thesit was used to extract enzymes from freshly prepared particulate enzyme fraction of M. luteus (13). Solubilized enzymes were stabilized in 20 mM magnesium acetate−15% glycerol−2 mM 2-mercaptoethanol and stored at −20°C.

Elongation products were run on polyacrylamide gels and stained by using the periodic acid−Schiff reagent (17). The reaction product was used for filter assay with [14C]glucose and acceptor teichuronic acid to determine the reaction product (RF = 0.0) from residual substrate (RF = 0.2) by the solvent system of isobutyric acid−1 M NH4OH (5:3, vol/vol) (4, 6). Assays for teichuronic acid synthesis also included UDP-ManNAcA in the incubation mixtures. Segments of the paper chromatograms which included the immobilized product were immersed in Ecolume (ICN Biomedicals Inc.), and the radioactivity was quantified by liquid scintillation counting.

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Polyacrylamide gel electrophoresis. Polyacrylamide gels were prepared and visualized as described in the accompanying paper (16), by using alcian blue and photochemical silver staining. Teichuronic acid samples of 1 to 10 μg were applied to each lane. Duplicate gels prepared for autoradiography were treated in 3% glycerol for 1 h prior to desiccation and exposure of Kodak X-Omat A film for 2 to 3 weeks.

Analytical methods. The protein content was determined by the bicinchoninic acid procedure (11) with bovine serum albumin as the standard. Neutral hexose was determined by an anthrone procedure (12) with glucose as the standard.

RESULTS

Optimal assay conditions with teichuronic acid acceptor. TheS-l-solubilized preparations of cytoplasmic membrane fragments of M. luteus contain both glucosyltransferase and the N-acetlylmannosaminurinosyltransferase, which cooperatively catalyze the synthesis of teichuronic acid. If UDP-[14C]glucose is the only sugar nucleotide supplied, the glucosyltransferase transfers a single glucosyl residue to each available teichuronic acid polymer chain with a suitable acceptor site. Under these circumstances the amount of [14C]glucose incorporated is a measure of the glucosyltransferase activity. The glucosyltransferase displayed optimal activity at pH values in the range of 8 to 9 and in the presence of 20 mM magnesium ion. Routine assays were conducted at pH 8.2 with 20 mM magnesium ion. These values are in agreement with the results for de novo synthesis of teichuronic acid by cytoplasmic membrane fragments (8).

The effect of substrate concentration on glucosyltransferase activity was determined from initial reaction velocities of [14C]glucose incorporation (Fig. 1). The initial reaction rates were determined from the first 10 min of reaction, although incorporation was linear for nearly 1 h following addition of UDP-[14C]glucose. Maximum reaction velocities were obtained at or above 0.4 mM UDP-glucose (Fig. 1A) and 50 mg of teichuronic acid per ml (Fig. 1B). These data fit expected kinetic theory, with a K_m for UDP-glucose of 2.9 × 10^-2 M^-1 and an apparent K_m for teichuronic acid of about 5 × 10^-2 M^-1. The latter assumes the average chain length of teichuronic acid to be 22 disaccharide repeat units (based on chain length distribution observed by polyacrylamide gel electrophoresis [16]).

Teichuronic acid as acceptor. During the release of teichuronic acid from cell walls, longer mild acid treatment results in a shift from a range of high-molecular-weight polymers (n = 25 to 45) to that of low-molecular-weight polymer (n = 10 to 25) (16). Analysis of these samples by proton nuclear magnetic resonance spectroscopy demonstrated that in addition to the release of teichuronic acid, prolonged acid treatment gradually solubilized peptidoglycan (data not shown). These fractions were also tested for their ability to serve as an acceptor for the incorporation of glucose. The specific acceptor activity (defined as picomoles of [14C] glucose incorporated per minute per gram of acceptor teichuronic acid) for incorporation of glucose was greatest in the 0- to 30- and 30- to 60-min fractions and decreased with longer duration of mild acid treatment of cell walls (Table 1). This decrease in specific acceptor activity indicates that teichuronic acid is released from cell walls mainly during the initial 60 min of treatment and that material released by more extensive treatment contains less teichuronic acid and more peptidoglycan fragments. The cell wall residue remaining after 120 min of acid treatment was further digested with lysozyme and subsequently analyzed for acceptor activity. Less than 5% of the solubilized material recovered was identified as having teichuronic acid acceptor activity. The most active acceptor fractions, released in the 0- to 30- and 30- to 60-min acid treatment intervals, were routinely used for the biosynthetic studies reported herein.

Biosynthetic elongation of teichuronic acid. To demonstrate that teichuronic acid serves as the acceptor for the glucosyltransferase as well as for the N-acetylmannosaminurinosyltransferase, we measured the incorporation of [14C]glucose into teichuronic acid as a function of time in both the absence and presence of UDP-ManNAcA (Fig. 2). Incubation of solubilized enzymes with teichuronic acid and UDP-[14C]glucose showed a small but measurable incorporation of [14C]glucose, which reached an essentially constant level after 2 h. This indicates the addition of a single glucose

![FIG. 1. Effect of substrate concentration on the initial rate of glucose incorporation by glucosyltransferase. Teichuronic acid used as the acceptor was released from cell walls by a 30- to 60-min acid treatment. Except as otherwise indicated, reaction mixtures contained 0.6 mM UDP-[14C]glucose, 87 mg of teichuronic acid per ml, and 0.2 mg of protein per ml of an ammonium sulfate cut of the Thesit extract of cytoplasmic membrane fragments as the source of glucosyltransferase. The reaction velocity is shown as a function of the concentration of UDP-glucose (A) and teichuronic acid (B).](http://jb.asm.org/)
residue to all available acceptor sites on teichuronic acid. In contrast, when UDP-ManNAcA was also present in the incubation mixture, the level of incorporation was three- to fivefold greater, indicating that additional acceptor sites for glucosyl residues were made available by the incorporation of ManNAcA residues. This result does not reveal the extent of elongation. It is possible that a substantial portion of the teichuronic acid polymers are not suitable acceptors for the glucosyltransferase because the terminal residue is already a glucosyl residue. When UDP-ManNAcA is added to the reaction mixture, the N-acetylmannosamuronosyltransferase effects the addition of a single ManNAcA residue to the nonreducing end of the polymer, thus converting it to a suitable acceptor for [14C]glucose incorporation. Alternatively, there might be repetitive sequential addition of both glucosyl and ManNAcA residues so that several disaccharide repeat units are added to some teichuronic acid chains.

The products of the above incorporation reactions were examined by polyacrylamide gel electrophoresis followed by autoradiography to determine whether any differences in polymer length could be detected (Fig. 3). The observed ladder of bands, which is typical of teichuronic acid, indicates that a broad range of teichuronic acid polymer chain lengths are suitable acceptors for the glucosyltransferase (lane 3). Inclusion of UDP-ManNAcA in the reaction mixtures led to the expected increase in the intensity of the banding pattern, but did not show any detectable shift in the position of the ladder of bands (compare lanes 1 and 2). This indicates that the number of moles of added disaccharide repeat units was small relative to the number of moles of acceptor teichuronic acid present. Therefore, in this experiment no appreciable increase in chain length was observed. The similarity of the binding pattern seen in the autoradiogram to that observed by silver staining indicated that all lengths of teichuronic acid serve as the acceptor.

To establish conclusively that teichuronic acid is being elongated, limiting concentrations of low-molecular-weight teichuronic acid were used in the transferase reaction mixtures. The material used had been fractionated on a Bio-Gel P-30 column and was from a fraction in which three polymer chain lengths predominated. These low-molecular-weight teichuronic acids contained 12 to 14 disaccharide repeat units (16). The amount of acceptor teichuronic acid added was diminished so that the ratio of substrate to acceptor would favor extensive elongation. Figure 4 shows the extent

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**TABLE 1. Specific acceptor activity of teichuronic acid released from cell walls over time**

<table>
<thead>
<tr>
<th>Duration of acid treatment (min)</th>
<th>Acceptor sp act (pmol/min per g)</th>
</tr>
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<tbody>
<tr>
<td>0-30</td>
<td>195 ± 45</td>
</tr>
<tr>
<td>30-60</td>
<td>193 ± 31</td>
</tr>
<tr>
<td>60-90</td>
<td>136 ± 31</td>
</tr>
<tr>
<td>90-120</td>
<td>82 ± 18</td>
</tr>
<tr>
<td>120-150</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>150-1,200</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Release was conducted at 90°C at 8 mg of cell walls per ml 50 mM HCl.
* b Cell walls were sequentially treated with acid for the indicated periods. The cell wall suspension was rapidly cooled, neutralized with 2 M ammonium hydroxide, and centrifuged to sediment the cell walls, which were then resuspended in acid for continued treatment. Each of the successive supernatant solutions was dialyzed and lyophilized to recover the material which was assayed for teichuronic acid acceptor activity.
* c Specific acceptor activity was expressed as picomoles of [14C]glucose incorporated per minute per gram of acceptor teichuronic acid. Values reported are the mean of three replicates of the experiment ± standard deviation.

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**FIG. 2.** Time course of incorporation of [14C]glucose into high-molecular-weight teichuronic acid (2 mg/ml). Assay mixtures contained 0.75 mM UDP-[14C]glucose, 2 mg of teichuronic acid (the 0- to 60-min fraction) per ml, and 1.25 mg of enzyme preparation (Thesit-activated cytoplasmic membrane fraction) per ml in the presence (●) or absence (○) of 0.6 mM UDP-ManNAcA. The acceptor teichuronic acid, which had been released from cell walls by a 0- to 60-min acid treatment, contained polymers ranging in length from 15 to 45 disaccharide repeat units.

**FIG. 3.** Incorporation of [14C]glucose into high-molecular-weight teichuronic acid. The figure shows an autoradiogram of a polyacrylamide gel separating teichuronic acid samples prepared in the experiment depicted in Fig. 2. Lanes: 1, 1-h incubation in the presence of UDP-ManNAcA; 2, 24-h incubation in the presence of UDP-ManNAcA; 3, 24-h incubation in the presence of UDP-ManNAcA.
of incorporation of [14C]glucose for three different acceptor concentrations as a function of incubation time. With UDP-[14C]glucose as the only sugar nucleotide present, the incorporation of glucose soon reached plateau levels which were proportional to the amount of teichuronic acid added as acceptor. Presumably all available acceptor sites were glucosylated early in the reaction, leading to this plateau. In contrast, when UDP-ManNAcA was also present in the incubation mixtures, the incorporation of glucose was linear with incubation time and the extent was 10- to 15-fold greater than in the absence of UDP-ManNAcA. This result is indicative of repetitive addition of disaccharide repeat units resulting in substantial elongation of the polymer chains.

Samples from these reactions were further analyzed by polyacrylamide gel electrophoresis and photochemical silver staining (Fig. 5A). Both exogenous acceptor teichuronic acid and elongated product teichuronic acid were detected by this means. Samples from the reaction containing UDP-[14C]glucose as the only sugar nucleotide showed no significant change in the intensity or the position of the ladder of bands as a function of incubation time (odd-numbered lanes). In contrast, reactions containing both UDP-[14C]glucose and UDP-ManNAcA showed a gradual shift toward longer polymer chains as the incubation progressed (even-numbered lanes). When acceptor teichuronic acid was most limiting, the ladder of bands was shifted by an average of 14 bands (lane 12). This indicates that all teichuronic acid had been elongated by an average of 14 disaccharide repeat units during the 24-h incubation.

Figure 5B shows the results of the same experiment as detected by autoradiography. In this instance, teichuronic acid was visualized only if it incorporated [14C]glucose. Incorporation of multiple residues contributed to a proportionate increase in band intensity. Samples from the reaction containing UDP-[14C]glucose as the only sugar nucleotide showed no significant change in either the intensity or the position of the banding pattern as a function of incubation time (odd-numbered lanes). In contrast, reaction mixtures containing both UDP-[14C]glucose and UDP-ManNAcA demonstrated both an increase in intensity and a shift in the position of the ladder of bands (even-numbered lanes). When acceptor teichuronic acid was most limiting, the autoradiogram shows addition of up to 17 disaccharide repeat units (compare lanes 11 and 12). These results (Fig. 5A and B) show clearly that incubation with only UDP-glucose leads to addition of a single glucose residue to acceptor teichuronic acid, whereas incubation with both UDP-glucose and UDP-ManNAcA permits extensive elongation of the acceptor by multiple additions of the disaccharide repeat units. Within the limits of experimental detection, all polymer lengths of teichuronic acid serve as acceptor.
DISCUSSION

The detergent-solubilized preparation of glucosyl- and N-acetylmannosaminuronosyltransferases described above has been shown to elongate teichuronic acid polymers by multiple additions of the ManNAcA-glucose disaccharide repeat unit. These findings corroborate and extend our previous findings with wall-membrane enzyme preparations and wall-bound teichuronic acid (15).

The release of teichuronic acid from cell walls requires considerable care to ensure high yields of active receptor. The acceptor of highest specific activity (based on total mass) is released during the first hour of acid treatment of cell walls. Nearly all of the acceptor activity was removed from the insoluble cell wall residue within 2 h of treatment.

Other experiments concerned with the effect of acid-catalyzed fragmentation of purified teichuronic acid on acceptor activity (data not shown) indicated that specific acceptor activity remained constant despite the fragmentation of teichuronic acid. This suggests that the decrease in specific activity observed for the material released from cell walls during sequential treatment stages is due to dilution by increased amounts of solubilized peptidoglycan which does not serve as an acceptor. This prompts the question of what sites on the acceptor are recognized by the enzymes during elongation of teichuronic acid. Since teichuronic acid specific acceptor activity is not increased with fragmentation (despite assumed liberation of additional sites for elongation), one envisions the involvement of the reducing end (or linkage region) as well as the site of elongation. Modification of the reducing end by β-elimination or aniline derivatization, however, has no measurable effect on incorporation of [14C]glucose during biosynthesis. The exact mechanism of elongation and recognition of acceptor sites remain to be elucidated.

Incorporation of [14C]glucose by both high- and low-molecular-weight teichuronic acid demonstrates that all lengths of polymer serve as acceptor. Quantitation of incorporation based on radioactivity (Fig. 4) indicates that several disaccharide repeat units must have been added to each acceptor. Unequivocal visual confirmation was achieved by demonstration of the polymer length distribution by electrophoresis on polyacrylamide gels. The gels show that all teichuronic acid polymers detectable by the electrophoretic procedure served as acceptors for incorporation of glucose and that these acceptors could be substantially extended in polymer size when the ratio of substrates to acceptors was favorable for multiple addition of disaccharide repeat units. This demonstration was made possible by the availability of a fraction of low-molecular-weight acceptor teichuronic acid which contained relatively few polymer lengths that could be distinctly resolved by electrophoresis.

Careful inspection of the autoradiogram of Fig. 5B shows faint bands of low-molecular-weight teichuronic acid only in reactions containing UDP-ManNAcA. These faint radioactive bands appear to belong to the same ladder of bands as the primary radioactive products and hence are probably teichuronic acid oligomers having fewer disaccharide repeat units. These minor bands may have resulted from fragmentation of labeled teichuronic acid during the incubation or during preparation of the sample for electrophoresis.

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LITERATURE CITED


