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Biosynthesis of D-Alanyl-Lipoteichoic Acid: Characterization of Ester-Linked D-Alanine in the In Vitro-Synthesized Product

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D-Alanyl-lipoteichoic acid (D-alanyl-LTA) contains D-alanine ester residues which control the ability of this polymer to chelate Mg^{2+}. In *Lactobacillus casei* a two-step in vitro reaction sequence catalyzed by the D-alanine-activating enzyme and D-alanine:membrane acceptor ligase incorporates D-alanine into membrane acceptor. In this paper we provide additional evidence that the in vitro system catalyzes the covalent incorporation of D-[^{14}C]alanine into membrane acceptor which is the poly([^{3}H]glycerol phosphate) moiety of D-alanyl-LTA. This conclusion was supported by the observation that the D-[^{14}C]alanine and [^{3}H]glycerol labels of the partially purified product were co-precipitated by antiserum containing globulins specific for poly(glycerol phosphate). The isolation of D-[^{14}C]alanyl-[^{3}H]glycerol from D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA synthesized in the in vitro system indicated that the D-alanine was linked to the poly(glycerol phosphate) chain of the LTA. A comparison of the reactivities of the D-alanine residues of D-alanyl-glycerol and D-alanyl-LTA supported the conclusion that the incorporated residue of D-alanine was attached by an ester linkage. Thus, the data indicated that the in vitro system catalyzes the incorporation of D-alanine covalently linked by ester linkages to the glycerol moieties of the poly(glycerol phosphate) chains of D-alanyl-LTA. New procedures are presented for the partial purification of D-alanyl-LTA with a high yield of ester-linked D-alanine and for the sequential degradation of the poly(glycerol phosphate) moiety substituted with D-alanine of D-alanyl-LTA with phosphodiesterase II/phosphatase from *Aspergillus niger*.

Membrane teichoic acids are linear polymers of poly(glycerol phosphate) covalently linked to glycolipid. These lipid-linked polymers, lipoteichoic acids (LTA) (39–41), are found in most gram-positive bacteria (11, 21, 31). The poly(glycerol phosphate) portion of LTA is selectively acylated with D-alanine ester residues, and in some cases it is also substituted with glycosyl groups. Recent findings indicate that LTA plays an important functional role in a variety of cellular processes. These include assembly of wall polymers (5, 13, 16), regulation of autolytic activity (9, 10), and control of the Mg^{2+} ion concentration for membrane-associated enzymes (1, 21, 28). In at least one of these functions, the D-alanine ester residues appear to have an important role in modulating the activity of the LTA. Baddiley and co-workers (1, 17, 18, 20) have proposed that the negatively charged polymer serves to chelate Mg^{2+} and therefore, to maintain a high concentration of this cation near the membrane. The capacity of the polymer to chelate Mg^{2+} is regulated in part by the D-alanine ester residues which neutralize the anionic nature of the phosphodiester links. Thus, the controlled incorporation of D-alanine into this polymer may be an important feature regulating its function.

In *Lactobacillus casei* the incorporation of D-alanine into LTA is accomplished in the following two-step reaction sequence (2, 22, 29, 32):

$$\text{enzyme} + \text{D-alanine} + \text{ATP} \rightleftharpoons \text{enzyme}\cdot\text{AMP-D-alanine} + PP_i \quad (1)$$

$$\text{enzyme}\cdot\text{AMP-D-alanine} + \text{membrane acceptor} \xrightarrow{\text{ligase}} \text{D-alanyl-membrane acceptor} + \text{enzyme} + \text{AMP} \quad (2)$$

In reaction 1, D-alanine is activated in the presence of ATP and the D-alanine-activating enzyme to form an enzyme·AMP-D-alanine complex with the release of PP_i. In reaction 2, the activated D-alanine is covalently linked to membrane acceptor in the presence of the D-alanine:membrane acceptor ligase. The precise function of the ligase has not been established.

The membrane acceptor in the in vitro system purified from *L. casei* has been assumed to be LTA. A variety of experiments have supported this assumption; i.e., the incorporated D-alanine

was covalently associated with LTA. However, these experiments did not provide convincing evidence that the incorporated D-alanine was covalently linked to the glycerol moieties of the poly(glycerol phosphate) of the LTA. It was the purpose of the experiments described below to provide definitive evidence that the in vitro system catalyzes the incorporation of D-alanine covalently linked to the poly(glycerol phosphate) moiety of D-alanyl-LTA. Because of the emphasis on the ester-linked D-alanine of LTA, this polymer is referred to as D-alanyl-LTA in this paper.

MATERIALS AND METHODS

Materials. We are indebted to Eugene Kennedy for a generous gift of phosphodiesterase II from *Aspergillus niger* (33) and to Rosemary Linzer and Hutton Slade for a generous gift of antiserum containing globulins specific for the poly(glycerol phosphate) moiety of D-alanyl-LTA. This antiserum was prepared against cells of *Streptococcus mutans* strain Ingbritt (1B) (serotype c) (15). Immunodiffusion tests revealed a single precipitin band between LTA and this antiserum.

D-[^{14}C]alanine (40 mCi/mmol) and [2-^{3}H]glycerol (200 mCi/mmol) were purchased from Amersham-Searle. Wheat germ acid phosphatase was obtained from Worthington Biochemicals Corp., and octyl-β-D-glucopyranoside was purchased from Calbiochem. Membrane filters (pore size 0.45 μm) were obtained from Millipore Corp. Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), glycerokinase (EC 2.7.1.30), and NAD^+ were purchased from Boehringer Mannheim GmbH. Sepharose 6B, Sephacryl S-200, and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals. Bio-Gel P-2 and AG 50W-X8(H^+) were supplied by Bio-Rad Laboratories. All other materials were reagent grade.

Growth of [^{3}H]glycerol-labeled cells. *L. casei* ATCC 7469 labeled with [^{3}H]glycerol was grown at 37°C in a medium which contained 2% glucose, 2% peptone (Difco Laboratories), 1% yeast extract (Difco), 1% potassium acetate, and 0.5% NaH_2PO_4. In addition, the medium contained 0.02% $MgSO_4 \cdot 7H_2O$, 0.00075% $MnSO_4 \cdot H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.001% NaCl. Each 1 liter of medium contained 1 mCi of [^{3}H]glycerol. Cells were grown to late log phase and harvested. The cells (6 g [wet weight] from 1 liter) were then washed five times at 4°C with 50 ml of 5 mM piperazine-acetate buffer, pH 6.5. The final wash also contained 1 mM dithiothreitol. The pellet was frozen at −20°C.

Toluene treatment of [^{3}H]glycerol-labeled cells of *L. casei*. The procedure for preparing toluene-treated cells was adapted from previously published procedures (4, 26, 34). A solution of 0.6% (vol/vol) toluene was made in 20 mM Tris-hydrochloride (pH 7.2) containing 20 mM $MgCl_2$ and 1 mM dithiothreitol (TMD buffer) by sonicating the solution (25 ml) for 1 min at 100 W with a Braun-sonic 1510. Cells (3 g) of [^{3}H]glycerol-labeled *L. casei* were suspended in 12 ml of TMD buffer containing toluene and stirred rapidly at room temperature for 15 min. The sample was centrifuged at 3,000 × *g* for 10 min at 4°C. The cells were suspended in 6 ml of TMD buffer containing toluene and sedimented at 3,000 × *g* for 10 min. Then the pellet was suspended in 12 ml of TMD buffer without toluene (20 mg of protein per ml) and stored at −196°C.

Alanine incorporation assay. This assay measured the incorporation of D-[^{14}C]alanine into the toluene-treated cells of *L. casei* which were retained by a 0.45-μm-pore-size membrane filter. The reaction mixture contained 40 mM piperazine acetate buffer (pH 6.5), 33 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM ATP (neutralized with NaOH), 0.1 mM D-[^{14}C]alanine (40 mCi/mmol), and toluene-treated cells (100 μg of protein) in a total volume of 50 μl. After the mixture was incubated at 37°C, the reaction was diluted and terminated by adding 1 ml of ice-cold 5 mM Tris-hydrochloride (pH 6.5) buffer containing 10 mM $MgCl_2$. The diluted mixture was transferred to the membrane filter, and the filter was washed three times with 1 ml of Tris-$MgCl_2$ buffer per wash. The damp membrane filter containing the toluene-treated cells was dissolved in 1 ml of ethyl acetate in a scintillation vial. The amount of radioactivity was determined by the method of Patterson and Greene (30).

Isolation of D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA. The [^{3}H]glycerol-labeled cells of *L. casei* were suspended in 50 ml of the reaction mixture described above. The mixture contained 0.1 mM D-[^{14}C]alanine (20 mCi/mmol). The cells were incubated at 37°C for 40 min before the incorporation reaction was terminated with 30 ml of ice-cold Tris-$MgCl_2$ solution. After the cells were sedimented at 3,000 × *g* for 10 min, they were resuspended and washed twice with 30 ml of Tris-$MgCl_2$ solution. The sedimented cells were dried under a stream of N_2. To delipidate the cells, they were extracted with 200 ml of chloroform-methanol (2:1) by stirring at room temperature for 2 h (38). The suspension was centrifuged at 6,000 × *g* for 10 min, and the supernatant fraction was discarded. The extraction procedure was repeated for two additional times, for a total of three extractions with chloroform-methanol for 6 h.

The delipidated cells were dried under N_2 to remove chloroform and methanol before they were suspended in 36 ml of 1% octyl-β-D-glucopyranoside in 5 mM piperazine-acetate buffer, pH 6.5. They were extracted for 3 h at 4°C with stirring. After the extracted cells were removed by centrifugation, the supernatant fraction was centrifuged at 100,000 × *g* for 1 h. To remove the detergent, this supernatant fraction was dialyzed overnight at 4°C against 5 mM piperazine-acetate buffer, pH 6.5. The dialyzed fraction, which was concentrated, contained impure D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA and was used for the partial purification described in the legend to Fig. 2.

Precipitation of D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA by antibody specific for D-alanyl-LTA. Antiserum containing globulins specific for the poly(glycerol phosphate) moiety (15) of D-alanyl-LTA was used to precipitate the doubly labeled D-alanyl-LTA. The sample mixture contained partially purified D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA (^{3}H, 2,180 dpm; ^{14}C, 5,680 dpm) in 0.9% NaCl in

a total volume of 107 μl. Increasing amounts of the antiserum or rabbit serum were added, and the sample mixtures were maintained at 37° C for 1 h. They were then maintained at 4°C for 2 days. The samples were transferred to microfuge tubes (1.5 ml) in 1 ml of cold 0.9% NaCl. The complex was sedimented for 4 min with a Beckman microfuge B. The precipitate was washed with 1 ml of cold 0.9% NaCl three times, and the precipitate was monitored for radioactivity.

Purification of D-[^{14}C]alanyl-[^{3}H]glycerol. The partially purified D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA was degraded with phosphodiesterase II/phosphatase as described in the legend to Fig. 4. The undegraded D-alanyl-LTA and enzyme protein were removed on a DEAE-Sephadex column (0.6 by 3 cm). The fraction that was not retained by this column was applied to an AG 50W-X8 (Na^+) column (0.9 by 28 cm) equilibrated with 25 mM formate buffer, pH 4.5. Alanine and glycerol are not retained on this resin under these conditions. Before the gradient was begun, the column was washed with 25 ml of 25 mM phosphate buffer, pH 7.0. The column was developed with a linear gradient between 25 mM formate buffer (pH 4.5; 140 ml) and 0.25 M NH_4Cl in 25 mM formate buffer (pH 4.5; 140 ml). D-[^{14}C]alanyl-[^{3}H]glycerol eluted at 0.22 M NH_4Cl (25 mM formate buffer, pH 4.5). This fraction was concentrated and filtered on a Bio-Gel P-2 column (1 by 100 cm) in 25 mM formate buffer, pH 4.5. After the fraction containing D-[^{14}C]-alanyl-[^{3}H]glycerol was concentrated, it was stored at −20°C. This purification procedure was used for all samples of D-alanyl-glycerol.

Determination of the reactivity of the D-alanine ester linkage in D-alanyl-LTA and D-alanyl-glycerol by hydrolytic cleavage. For D-alanyl-LTA, the D-[^{14}C]alanine-labeled polymer (2,350 dpm) was added to 1 ml of 50 mM buffer at the indicated pH (see Table 2). For measuring the percent hydrolysis, the pH of the sample was adjusted to approximately 5 to 6 before the sample was applied to a DEAE-Sephadex column (0.6 by 2 cm). The undegraded D-[^{14}C]alanine-labeled D-alanyl-LTA was retained, whereas the D-[^{14}C]alanine was not retained. The column was washed three times with 1 ml of 50 mM formate buffer, pH 4.5. Each of the 1-ml fractions was assayed for radioactivity. The amount of D-alanyl-LTA remaining at a particular time was calculated from the initial amount of D-alanyl-LTA and the amount of D-alanine released.

For D-alanyl-glycerol, the D-[^{14}C]alanine-labeled compound (1,670 dpm) was added to 1 ml of 50 mM buffer at the indicated pH. For measuring percent hydrolysis, the pH of the sample was adjusted to 5 to 6, and the sample was applied to an AG 50W-X8 (Na^+) column (0.6 by 1 cm), which retained D-[^{14}C]alanyl-glycerol but not D-[^{14}C]alanine. After the column was washed with 3 ml of 50 mM formate buffer (pH 4.5), the unreacted D-[^{14}C]alanyl-glycerol was eluted with 4 ml of 0.5 M KCl in 50 mM formate buffer, pH 4.5. Fractions (1 ml) were collected and monitored for radioactivity. At each pH value, the amount of remaining D-alanyl-glycerol was determined at least four times during the reaction, and these values were used in the first-order plot from which the half-life ($t_{1/2}$) was calculated.

Analytical methods. Glycerol was determined by the method of Chernick (8). The fluorescence of NADH was measured with a Farrand Mark I spectrofluorometer. Phosphorus was analyzed by the method of Lowry et al. (24). The chromophore was measured with a Cary 17D spectrophotometer at 820 nm. Amino acids were analyzed with a Durrum amino acid analyzer (model D-500). For the analysis of glycerol in D-alanyl-glycerol, the compound was hydrolyzed in 0.1 M NaOH; for the amino acid analyses, the compounds were hydrolyzed in 25 mM formate buffer (pH 4.5) at 85°C.

Protein was determined by the method of Lowry et al. (25), using bovine serum albumin as the standard. Descending paper chromatography was performed on Whatman 3MM paper in a solvent that contained butanol, propionic acid, and water (142:71:100, vol/vol/vol) (V. Brautigan, Ph.D. thesis, Northwestern University, Evanston, Ill., 1977). For the determination of radioactivity on paper chromatograms, 2-cm sections were counted in a scintillation fluid that contained 0.3% 2,5-diphenyloxazole and 0.025% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene. For the determination of radioactivity in aqueous samples, the scintillation fluid described by Patterson and Greene (30) was used. Radioactivity was measured with a Packard model 2425 Tri-Carb liquid scintillation spectrometer.

RESULTS

D-[^{14}C]alanine incorporation into [^{3}H]-glycerol-labeled *L. casei*. *L. casei* was chosen for this investigation because it contains a high concentration of the D-alanine-activating enzyme (2). In addition, the membrane of this organism contains a well-characterized D-alanyl-LTA (19, 27, 36, 41) and does not contain a wall teichoic acid (19). For the initial experiments on the incorporation system (22, 32), membranes were used as a source of the membrane acceptor in conjunction with the D-alanine-activating enzyme and D-alanine:membrane acceptor ligase. To have a system with higher activity as well as have [^{3}H]glycerol-labeled membrane acceptor, we examined toluene-treated cells for their ability to incorporate D-[^{14}C]alanine.

As Fig. 1 shows, toluene-treated cells of *L. casei* showed a rapid incorporation of D-[^{14}C]-alanine when they were incubated under optimal conditions. The incorporation of D-alanine was dependent on ATP and was essentially complete after 30 min. As would be expected for incorporation into D-alanyl-LTA (32), the incorporated D-[^{14}C]alanine was released upon addition of 0.83 M hydroxylamine ($t_{1/2}$, 3 min). The higher specific activity of the system, coupled with the ability to label the membrane acceptor with [^{3}H]glycerol, supported the decision to use toluene-treated cells of *L. casei* for this investigation.

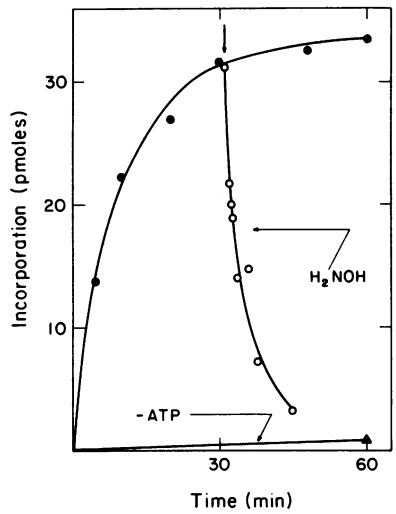

FIG. 1. *Incorporation of D-[^{14}C]alanine into toluene-treated cells of L. casei. The alanine incorporation assay used is described in the text. Symbols: ○, 0.83 M H_2NOH (pH 7.0) added at the arrow; ▲, ATP omitted from the assay.*

Isolation and purification of D-[^{14}C]alanine•[^{3}H]glycerol-labeled D-alanyl-LTA. Many of the published procedures for the isolation of D-alanyl-LTA cause the hydrolysis of the D-alanine ester residues from the polymer. For example, one of the standard procedures that utilizes extraction with 80% aqueous phenol causes a large loss in D-alanine (12). The relatively mild procedure described below utilizes the delipidation procedure of Wicken et al. (38) and an extraction procedure using octyl-β-D-glucopyranoside at 4°C. Extractions with this detergent gave a 10-fold-higher yield of crude D-alanyl-LTA than the yield achieved by water extractions at 37°C as recommended by Wicken and Knox (41). Water extractions at higher temperatures gave D-alanyl-LTA with a low yield of alanine.

As Fig. 2A shows, gel filtration of the octyl-β-D-glucopyranoside extract from delipidated cells on Sepharose 6B gave three labeled fractions. Fraction 1, which eluted near the void volume, has been characterized by Button and Hemmings (7) and Cleveland et al. (9) as fully acylated, micellar LTA. Fraction 2 was included in the gel and eluted at a position frequently associated with deacylated or partially deacylated LTA. Fraction 3, at the total inclusion volume, contained free D-[^{14}C]alanine.

To dissociate the D-alanyl-LTA micelle in fraction 1, filtration in the presence of 0.5% octyl-β-D-glucopyranoside and 1 M LiCl was performed. The combination of a detergent with a high critical micelle concentration (0.73%) (3) and LiCl provided the conditions necessary to break up the D-alanyl-LTA micelle without deacylating the polymer. Filtration of fraction 1 on Sepharose 6B in this medium caused both D-[^{14}C]alanine and [^{3}H]glycerol labels to elute slightly before the total inclusion volume, below the resolving ability of this column. Resolution was achieved by using Sephacryl S-200 under the same conditions. As Fig. 2B shows, a fraction of the [^{3}H]glycerol label was now resolved from the D-[^{14}C]alanine label. However, a significant fraction of the [^{3}H]glycerol label coeluted with the D-[^{14}C]alanine label. We concluded that this fraction contained D-[^{14}C]alanine•[^{3}H]glycerol-labeled acylated LTA in nonmicellar form. After the detergent and 1 M LiCl were removed from this fraction, it was applied to a Sepharose 6B column (Fig. 2A, inset). A single fraction, which eluted at the void volume, was observed and was assumed to be the partially purified, D-[^{14}C]alanine•[^{3}H]glycerol-labeled micellar, acylated LTA. Our goals were to use this partially purified D-alanyl-LTA for the isolation of the moiety to which D-[^{14}C]alanine is covalently linked and to establish that the antibody anti-LTA [poly(glycerol phosphate)] (15) coprecipitates both the [^{3}H]glycerol label and the D-[^{14}C]alanine label.

Simultaneous precipitation of D-[^{14}C]alanine and [^{3}H]glycerol labels of D-alanyl-LTA. Preliminary experiments (29) provided evidence that two antisera containing globulins specific for poly(glycerol phosphate) precipitate D-alanyl-membrane acceptor that has been solubilized with Triton X-100. However, no attempt was made to purify the acceptor or to show coprecipitation of the D-[^{14}C]alanine and [^{3}H]glycerol labels.

Simultaneous precipitation of the D-[^{14}C]alanine and [^{3}H]glycerol labels of the partially purified D-alanyl-LTA would further support the conclusion that the D-alanine was incorporated into D-alanyl-LTA. As Fig. 3 shows, the [^{3}H]-glycerol and D-[^{14}C]alanine labels were co-precipitated by antiserum containing globulins specific for poly(glycerol phosphate) (15). Immunodiffusion tests revealed a single precipitin band between D-alanyl-LTA and this antiserum. The similar ratios of the two labels at the different concentrations of antiserum indicated that the two labeled compounds were both associated with the same substance, presumably D-alanyl-LTA. Although these data suggested that the incorporated D-alanine was covalently linked to the poly(glycerol phosphate) moiety of D-alanyl-LTA, they did not prove it.

Degradation of D-[^{14}C]alanine•[^{3}H]glyc-

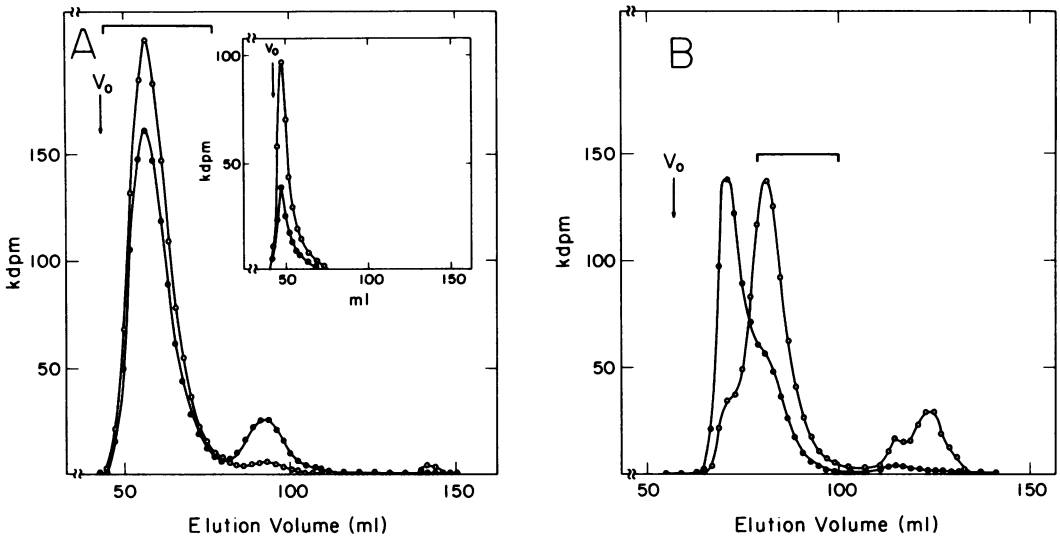

FIG. 2. *Partial purification of D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA. (A) Gel filtration on Sepharose 6B. The column (1.5 by 90 cm) was eluted with 5 mM piperazine hydrochloride, pH 6.5. Each fraction was assayed for radioactivity (10^3 disintegrations per minute per fraction) from D-[^{14}C]alanine (○) and from [^{3}H]glycerol (●). (B) Gel filtration on Sephacryl S-200 in the presence of 0.5% octyl-β-D-glucopyranoside containing 1 M LiCl in 5 mM piperazine hydrochloride, pH 6.5. The fraction marked by the bar in (A) was concentrated and applied to a column (1.5 by 90 cm). The column was eluted with this buffer, and each fraction was assayed for radioactivity. The fraction marked with a bar was combined and filtered on a Sephadex G-25 column to remove the detergent and LiCl. The partially purified D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA was filtered again on Sepharose 6B in the absence of detergent and LiCl (A, inset).*

erol-labeled D-alanyl-LTA by phosphodiesterase II/phosphatase. In 1978, Schneider and Kennedy (33) described a novel phosphodiesterase from *A. niger* which attacks glycerophosphodiester bonds with the liberation of glycerol. If this enzyme tolerates D-alanyl substitution of the glycerol phosphate moieties of D-alanyl-LTA, it may be possible to degrade sequentially the poly(glycerol phosphate) in the presence of phosphatase, with the liberation of the postulated D-alanyl-glycerol.

The degradation of D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA is shown in Fig. 4. By using phosphodiesterase II in the presence of wheat germ acid phosphatase, it was possible to follow the appearance of the D-[^{14}C]alanine and [^{3}H]glycerol labels as D-alanyl-LTA was degraded. The labeled degradation products were not retained by DEAE-Sephadex, whereas the D-alanyl-LTA was retained. The optimal pH of 4.5 recommended by Schneider and Kennedy (33) for phosphodiesterase II was used. The time course of this reaction was long. After 138 h, 50% of the [^{3}H]glycerol label was released. The data in Fig. 4 were corrected for the nonenzymic release of D-[^{14}C]alanine from the undegraded D-alanyl-poly(glycerol phosphate) of D-alanyl-LTA. Because of the lability of the D-alanyl-glycerol, a fraction of the D-[^{14}C]alanine label was free D-[^{14}C]alanine that was derived from the hydrolysis of the degradation product D-alanyl-glycerol. The ratio of D-[^{14}C]alanine to [^{3}H]glycerol (3.1 ± 0.3) in Fig. 4 was constant throughout the time course. This ratio did not reflect a molar ratio and varied among our preparations of D-alanyl-LTA. Continued degradation of the D-alanyl-LTA did not improve the yield of D-alanyl-glycerol due to the lability of both the D-alanyl-LTA and the D-alanyl-glycerol.

Isolation, purification, and characterization of D-alanyl-glycerol. Paper chromatography of the reaction mixture provided evidence for a new compound resulting from the degradation of D-alanyl-LTA. As Fig. 5 shows, incubation of D-[^{14}C]alanine-labeled D-alanyl-LTA with phosphodiesterase II/phosphatase resulted in a loss of D-alanyl-LTA, as measured by origin material, and was accompanied by the appearance of D-[^{14}C]alanine (R_f = 0.35) and a new compound (R_f = 0.50) containing D-[^{14}C]alanine. As described below, this compound was identified as D-alanyl-glycerol.

To characterize this compound, a larger amount was isolated from enzymically degraded D-alanyl-LTA, as described above. The reaction

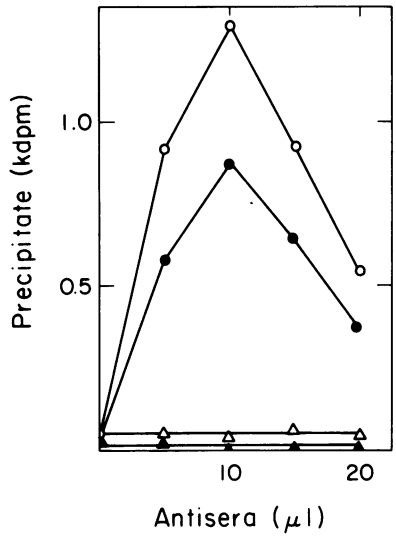

FIG. 3. *Precipitation of D-[^{14}C]alanine-[^{3}H]glycerol-labeled D-alanyl-LTA by antiserum containing globulins specific for poly(glycerol phosphate). The precipitation assay is described in the text and was performed with rabbit antiserum prepared against S. mutans Ingbritt* (○ *and* ●). *As a control* (△ *and* ▲), *rabbit serum was used. Radioactivity (10^{3} disintegrations per minute per precipitate) from D-[^{14}C]alanine* (○ *and* △) *and [^{3}H]glycerol* (● *and* ▲) *of the partially purified D-alanyl-LTA was measured.*

mixture was applied to a DEAE-Sephadex column to remove undegraded D-alanyl-LTA, as well as much of the enzyme protein. The effluent from this column was applied to a column of AG 50W-X8 (Na^+). D-[^{14}C]alanyl-[^{3}H]glycerol was retained by the column, whereas D-[^{14}C]alanine and [^{3}H]glycerol were not retained. Gradient elution of this column gave a single fraction containing both radioactive labels at 0.22 M NH_4Cl in 25 mM formate buffer, pH 4.5. Filtration of this labeled compound on Bio-Gel P-2 gave a labeled fraction with a K_{av} of 0.77. This positively charged, low-molecular-weight compound has a D-alanine ester linkage of high reactivity, as described below. An analysis of this compound gave a ratio of glycerol to alanine of 1.0 (Table 1). With the exception of slight traces of glycine and serine, no other amino acid was detected. There was no phosphorus present in the sample. Therefore, it was concluded that this compound, which appeared upon sequential degradation of D-alanyl-LTA with phosphodiesterase II/phosphatase, was D-alanyl-glycerol. For comparison, an analysis of D-alanyl-LTA that was isolated by our procedure is shown. The ratio of phosphorus to glycerol to alanine was 0.93:1.0:0.37 (Table 1).

Reactivity of the D-alanine ester linkage in D-alanyl-glycerol and D-alanyl-LTA. One of the features of D-alanyl-glycerol, which limited our ability to detect it, is its reactivity. At pH's above 6.0, it readily hydrolyzed to D-alanine and glycerol (Table 2). For example, the $t_{1/2}$ at pH 6.0 (37°C) was 8.8 h and decreased to 0.4 h at pH 9.0 (37°C). The $t_{1/2}$ of 166 h at pH 4.5 (37°C) was satisfactory for isolating the compound from D-alanyl-LTA with the phosphodiesterase II/phosphatase.

An interesting feature of D-alanyl-glycerol was shown in a comparison of the $t_{1/2}$ values of this compound and D-alanyl-LTA. For example, the $t_{1/2}$ for D-alanyl-glycerol was 8.8 h at pH 6.0 (37°C), whereas the $t_{1/2}$ for D-alanyl-LTA was more than 10,000 h. These differences were not immediately recognized and had previously hindered our attempts to isolate D-alanyl-glycerol from D-alanyl-LTA.

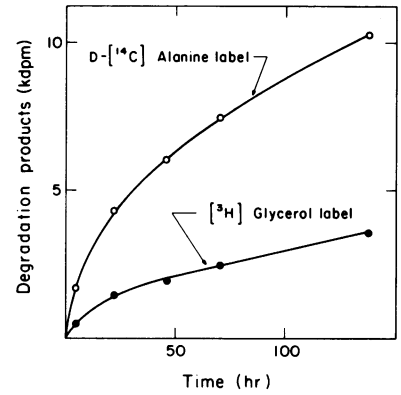

FIG. 4. *Formation of degradation products from D-[^{14}C]alanine-[^{3}H]glycerol-labeled D-alanyl-LTA by phosphodiesterase II/phosphatase. Symbols:* ○ *and* ●, *products with the D-[^{14}C]alanine and [^{3}H]-glycerol labels, respectively. The ^{14}C label was corrected for the D-[^{14}C]alanine that was hydrolyzed non-enzymically from the undegraded portions of the D-alanyl-LTA. The reaction mixture for a given time point contained D-[^{14}C]alanine-[^{3}H]glycerol-labeled D-alanyl-LTA (^{3}H, 6,800 dpm; ^{14}C, 18,200 dpm), 50 mM formate buffer (pH 4.5), 180 μg of phosphodiesterase II, and 330 μg of wheat germ acid phosphatase in a total volume of 760 μl. The reaction mixtures were incubated for the indicated times at 37°C. The degradation products were separated from the undegraded D-alanyl-LTA by retaining the polymer on DEAE-Sephadex. The sample was applied to this column (0.6 by 3 cm), and the column was eluted with 3 ml of 50 mM formate (pH 4.5) in 1-ml portions. These effluents were assayed for radioactivity. Although degradation of D-alanyl-LTA was observed with phosphodiesterase II, the addition of wheat germ acid phosphatase stimulated the rate of degradation. The activity with only phosphodiesterase II results from phosphatase in the enzyme preparation (E. Kennedy, personal communication).*

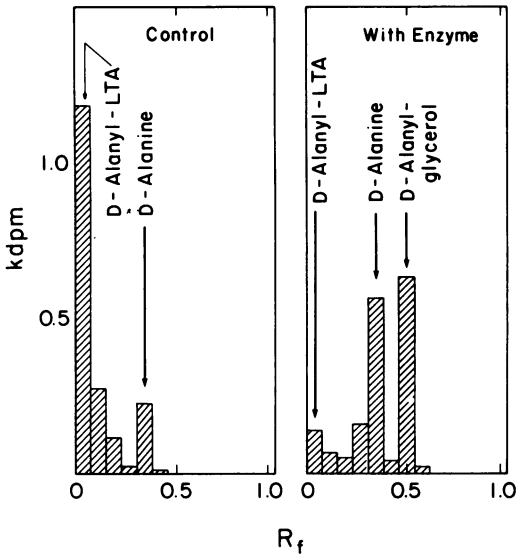

FIG. 5. *Analysis of a reaction mixture during the degradation of D-alanyl-LTA. Samples of D-[^{14}C]alanine-labeled D-alanyl-LTA (2,200 dpm) were incubated for 3 days at 37°C in 50 mM formate buffer (pH 4.5) with 180 μg of phosphodiesterase II and 330 μg of wheat germ acid phosphatase in a total volume of 500 μl. In the control, the enzymes were omitted, and the sample was incubated for 3 days at 37° C. Samples were analyzed by paper chromatography, and the radioactivity was measured as described in the text.*

TABLE 1. *Analyses of D-alanyl-LTA and D-alanyl-glycerol*

Compound	Analysis[a]		
	Phosphorus	Glycerol	Alanine
D-Alanyl-LTA	0.93	1.0[b]	0.37[b]
D-Alanyl-glycerol	0	1.0	0.99

[a] These analyses are described in the text (ratio to glycerol).

[b] The specific activities of D-[^{14}C]alanine and [^{3}H]-glycerol were calculated from isolated D-[^{14}C]alanyl-[^{3}H]glycerol. These activities were used to calculate the D-alanine and glycerol contents of the partially purified D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA described in the legend to Fig. 2.

DISCUSSION

The D-[^{14}C]alanyl-membrane acceptor of the D-alanine incorporation system from *L. casei* was isolated as D-alanyl-LTA. From this LTA we isolated D-alanyl-glycerol by sequentially degrading the polymer with phosphodiesterase II/phosphatase. While this manuscript was in preparation, Fiedler (F. Fiedler, personal communication) also found that phosphodiesterase II/phosphatase from *A. niger* could be used to characterize D-alanyl-LTA. The structure of this degradation product was consistent with the structure of the intracellular glycerol teichoic acid established by Kelemen and Baddiley (19). In their assignment it was reasoned that D-alanine is joined to the polymer through ester linkages at position 2 of the glycerol residues. The facts that we have been unable to carry out an in vitro incubation with added membrane acceptor and that we have to rely on the endogenous membrane acceptor emphasize the importance of isolating and characterizing the incorporation product of the in vitro system. The isolation of D-alanyl-glycerol, the co-precipitation of the D-[^{14}C]alanine and [^{3}H]glycerol labels of LTA by anti-LTA, and the coelution of the two labels indicated that D-alanine is incorporated into an ester linkage on the glycerol of the poly(glycerol phosphate) chain of the LTA from this organism.

To minimize the loss of ester-linked D-[^{14}C]-alanine, mild procedures must be used for the extraction and purification of D-[^{14}C]alanine-labeled D-alanyl-LTA. We describe above a procedure for the partial purification of D-alanyl-LTA in high yields that retain a high proportion of the D-alanyl ester residues. Several procedures for the preparation of LTA which use phenol extraction, have been reported in the literature, and they give LTA with low yields of ester-linked D-alanine (7, 12, 21, 38). One exception is the procedure described by Silvestri et al. (37), which gives D-alanyl-LTA with a high yield of alanine. In our procedure, the combination of octyl-β-D-glucopyranoside and LiCl provides a

TABLE 2. *pH stability of D-alanyl-LTA and D-alanyl-glycerol*

pH[a]	Temp (°C)	$t_{1/2}$ (h)[b]	
		D-Alanyl-glycerol	D-Alanyl-LTA
2.0	37	427	91.2
4.5	37	166	281
6.0	37	8.8	>10,000[c]
8.0	37	0.8	3.9
9.0	37	0.4	0.9
4.5	4	4,400[d]	5,700[d]
4.5	85	1.9	6.2

[a] Buffers were as follows: pH 2.0 and 4.5, 50 mM formate; pH 6.0 and 8.0, 50 mM phosphate; pH 9.0, 50 mM Tris-hydrochloride.

[b] Values for $t_{1/2}$ were calculated from first-order plots.

[c] The compound was 95 to 100% stable after 1,700 h.

[d] Estimated from first-order plots.

useful step for dissociating the D-alanyl-LTA micelle and removing a significant fraction of the glycerol that is not part of the D-alanyl-LTA without deacylating the polymer. The preparation of D-alanyl-LTA from *L. casei* purified by the procedure described here gave a ratio of phosphorus to glycerol to alanine of 0.93:1.0:0.37. Thus, this procedure gives a partially purified preparation of D-alanyl-LTA with a high yield of alanine.

It has been reported by Shabarova et al. (35) that adjacent phosphate and hydroxyl groups increase the reactivity of D-alanine ester linkages in model compounds to hydroxylamine at pH 7.4. This increased reactivity has contributed to the difficulty in isolating degradation compounds with ester-linked D-alanine from D-alanyl-LTA. In the experiments described here, we examined the stability of D-alanyl-LTA and D-alanyl-glycerol, and we conclude that pH 4.5 is the optimal pH for carrying out the enzymic degradation. At this pH, the $t_{1/2}$ for D-alanyl-glycerol was 166 h, and the $t_{1/2}$ for D-alanyl-LTA was 281 h. In addition, the pH optimum for phosphodiesterase II is 4.5 (33). Thus, these experimental conditions gave the optimal recovery of D-alanyl-glycerol from D-alanyl-LTA.

A comparison of the $t_{1/2}$ for D-alanyl-glycerol and the $t_{1/2}$ for D-alanyl-LTA as a function of pH reveals a different reactivity profile in the pH range from 2 to 9. This is consistent with different vicinal substituents in the two compounds. In the case of D-alanyl-LTA, the phosphodiester linkages provide adjacent anionic groups in this pH range. In D-alanyl-glycerol, vicinal hydroxyl groups are adjacent to the D-alanine ester linkage. The high reactivity of the D-alanyl-glycerol at pH 6.0 relative to D-alanyl-LTA can be partially explained by the inductive effect of the vicinal hydroxyl groups, which increase the electrophilicity of the carbonyl carbon (6, 44). In addition, the hydroxyl group can also have an "internal solvation effect," in which reactivity is increased by hydrogen bonding of the oxygen atom of the carbonyl to the hydrogen of the hydroxyl group in the transition state (6). Because D-alanyl-LTA has vicinal phosphodiester groups, this feature does not contribute to the reactivity of the D-alanine ester linkage of the polymer in the pH range from 4.5 to 6.0. In addition, the anionic phosphodiester groups of the D-alanyl-LTA could also account for additional stability of ester-linked D-alanine on LTA in this range. Electrostatic attraction of the protonated amino group of the D-alanine residue by these anionic groups may also increase the stability. Moreover, the anionic phosphodiester linkages could shield the carbonyl carbon from nucleophilic attack by OH^- in the range where the concentration of this nucleophile is low (43). It is interesting to note that the phosphodiester anion becomes protonated in the pH range from 1 to 2, where the reactivity of D-alanyl-LTA becomes greater than D-alanyl-glycerol (Table 2).

One would expect transacylation in D-alanyl-glycerol similar to that observed for aminoacyl-tRNA (14, 23, 42). Thus, we are not certain whether the isolated compound is D-alanyl-1(3)-glycerol or D-alanyl-2-glycerol or an equilibrium mixture of these two molecules.

The sequential degradation of the poly(glycerol phosphate) moiety of purified D-alanyl-LTA by phosphodiesterase II/phosphatase provides a procedure for analyzing the distribution of the D-alanine ester residues in this moiety of the LTA. Before the availability of this enzyme, no procedure for sequentially degrading the poly(glycerol phosphate) with ester-linked D-alanine existed. Degradation of in vitro-synthesized D-[^{14}C]alanine·[^{3}H]glycerol-labeled D-alanyl-LTA with this enzyme showed that the D-[^{14}C]alanine label was distributed evenly in the poly(glycerol phosphate) moiety after a 40-min incubation with the labeled amino acid. The methodology described above provides an experimental approach for asking some fundamental questions concerning the mechanism of assembly of D-alanyl-LTA.

The results presented here, together with those reported by Neuhaus et al. (29), support the proposal that the in vitro incorporation system described by Reusch and Neuhaus (32) and Linzer and Neuhaus (22) catalyzes the acylation of the poly(glycerol phosphate) moiety of LTA by D-alanine. The selective acylation of LTA by D-alanine and presumably the selective deacylation of this membrane polymer are important mechanisms for modulating at least one of the functions of this polymer, the chelation of Mg^{2+}.

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