Biosynthesis of Membrane Teichoic Acid

A ROLE FOR THE D-ALANINE-ACTIVATING ENZYME*

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SUMMARY

A second protein, the stimulator, has been identified as a necessary component for the in vitro incorporation of D-alanine into membrane teichoic acid of Lactobacillus casei (ATCC 7469). The incorporation activity is dependent on membrane fragments, ATP, D-alanine:membrane acceptor ligase (Reusch, V. M., Jr., and Neuhaus, F. C. (1971) J. Biol. Chem. 246, 6136), and the stimulator. The stimulator and the D-alanine:membrane acceptor ligase differ significantly with respect to exclusion volume on Sephadex G-100, chromatography on DEAE-cellulose, heat lability, and sensitivity to p-hydroxymercuribenzoate. It is proposed that the stimulator is the D-alanine-activating enzyme previously described by Baddiley and Neuhaus ((1960) Biochem. J. 75, 579). The stimulator and the D-alanine-activating enzyme co-elute on Sephadex G-100 and on DEAE-cellulose at pH 0.0 and pH 7.8. They are inactivated to the same extent by incubation at 45° and by incubation in the presence of p-hydroxymercuribenzoate. Under assay conditions in which the stimulator concentration is limiting with respect to ligase, the D-alanine incorporation system has a high specificity for D-alanine. Each of these properties is also characteristic of the D-alanine-activating enzyme. A two-stage reaction sequence utilizing the activating enzyme and the ligase for the incorporation of D-alanine into membrane teichoic acid is suggested.

Membrane teichoic acids in gram-positive bacteria are characterized as linear poly(glycerolphosphate) polymers with D-alanine ester residues and, in some cases, glycosyl substituents (1, 2). They appear to associate with membrane glycolipid forming a complex called lipoteichoic acid (3, 4). A suggested function of membrane teichoic acid is to maintain a high concentration of divalent cations in the region of the membrane (5, 6, 6a). Baddiley and coworkers have proposed that this cationic-binding capacity is partially regulated by the D-alanine associated with the teichoic acid (7).

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The in vitro incorporation of D-[14C]alanine into membrane fragments of Lactobacillus casei (ATCC 7469) has been described (8). This incorporation is dependent on membrane fragments, ATP, and supernatant fraction. A necessary component of this fraction, the D-alanine:membrane acceptor ligase, has been partially purified and characterized (8). The product of this incorporation has properties that are characteristic of the alanine residues in the membrane teichoic acid of this organism (9).

It is the purpose of this communication to report the identification of a second protein required, in addition to the ligase, for the incorporation of D-alanine. This protein, designated stimulator, has properties similar to those reported by Baddiley and Neuhaus (10) for the D-alanine-activating enzyme. A two-step reaction sequence for the incorporation of D-alanine is proposed.

\[ \text{Enzyme} + \text{D-Alanine} + \text{ATP} \rightarrow \text{Enzyme-AMP-D-Alanine} + \text{PP}_1 \] (1)

\[ \text{Enzyme-AMP-D-Alanine} + \text{membrane acceptor} \xrightarrow{\text{ligase}} \text{D-alanyl-membrane acceptor} + \text{enzyme} + \text{AMP} \] (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 inorganic pyrophosphate. In Reaction 2, the activated D-alanine is covalently linked to membrane acceptor in the presence of the D-alanine:membrane acceptor ligase. A preliminary report of this work has been presented (11).

EXPERIMENTAL PROCEDURE

Materials—D-[14C]Alanine (uniformly labeled) with a specific activity of 40.9 mCi per mmole was purchased from Amersham-Searle. Sephadex G-25 and G-100 were obtained from Pharmacia. DEAE-cellulose (Whatman DE-52) was obtained from Reeve Angel. Membrane filters (MF type, 0.45-μ pore size) and filter holder apparatus were the products of Millipore Inorganic pyrophosphatase (EC 3.6.1.1.) (500 units per mg of protein) was obtained from Sigma in a lyophilized form.

Enzyme and Membrane Preparation—Extracts and membranes were prepared from L. casei (ATCC 7469) according to the method of Reusch and Neuhaus (8). After the disrupted cells were centrifuged at 100,000 × g for 90 min, the extract was centrifuged at 368,000 × g for 90 min to remove additional cell debris. The supernatant fraction was concentrated 2-fold.
using Sephadex G-25. Enzyme and membrane preparations were stored in a liquid nitrogen freezer.

Alanine Incorporation Assay—The assay measured the incorporation of n-[14C]alanine into membrane fragments which were retained by a 0.45-μm pore size membrane filter. The incorporation of alanine was dependent on both the n-alanine:membrane acceptor ligase and the stimulator (Table I). The reaction mixture contained the following components: 25 mM MgCl₂; 5 mM ATP (adjusted to pH 6.5 with NaOH); 30 mM piperazine-acetate, pH 6.5; 0.04 mM n-[14C]alanine (40.9 mCi per mmole); 170 μg of membrane fragments; and enzyme fractions as indicated in a total volume of 50 μl. The reaction mixture was incubated at 37° for 30 min. The reaction was terminated by the addition of 20 volumes of cold buffer (5 mM Tris-HCl, pH 6.5, and 10 mM MgCl₂) and maintained at 4° until filtered. The contents of the reaction tube were transferred to a 0.45-μm membrane filter, and the filter was washed with 10 ml of the cold buffer. The membrane filter containing labeled membranes was dissolved in 1.0 ml of ethyl acetate by either ligase or stimulator. The concentrations were chosen to be limiting with respect to the enzyme under study and saturating with respect to the second enzyme. A unit of n-alanine:membrane acceptor ligase is defined as the amount of enzyme required for the incorporation of 1 nmole of n-[14C]alanine per hour in the presence of a saturating concentration of stimulator. Conversely, a unit of stimulator is defined as the amount of enzyme required for the incorporation of 1 nmole of n-[14C]alanine per hour in the presence of a saturating concentration of n-alanine:membrane acceptor ligase.

Additional Assays—The activity of the n-alanine-activating enzyme was determined with the hydroxamate assay of Baddiley and Neuhaus (10). Alanine hydroxamic acid is formed by the reaction of hydroxylamine with the activated n-alanine complex (Reaction 1). The reaction mixture contained the following components: 100 mM MgCl₂; 10 mM ATP; 1 mM salt-free H₂N-OH (adjusted to pH 7.8); 100 mM n-alanine; 100 mM Tris-HCl, pH 7.8; and enzyme preparation in a total volume of 0.5 ml. The reaction mixture was incubated at 37° for 1 hour. The reaction was terminated and the amount of hydroxamic acid was determined by the method of Cormier and Novelli (12). A unit of n-alanine-activating enzyme is defined as that amount of enzyme required for the formation of 1 nmole of alanine hydroxamate per hour.

Control experiments were performed without added alanine to correct for the effects of chromogenic substances and endogenous activity. Since piperazine-acetate was used as one of the buffers in this work, the acetate-CoA ligase (AMP) (EC 6.2.1.1.) was also measured by the hydroxamate assay using 100 mM acetate as substrate instead of n-alanine.

Analytical Methods—Measurements of radioactivity were made in polyethylene vials with a Packard Tri-Carb liquid scintillation spectrometer (Model 314-EX). Samples were counted in 15 ml of the Triton-toluene (1:2) scintillation fluid described by Patterson and Greene (13) and evaluated by Benson (14). Protein concentrations were determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

Detection of Stimulator—The presence of an additional component that functions in the incorporation of n-alanine into membrane teichoic acid was considered in accounting for the large apparent loss of n-alanine:membrane acceptor ligase activity observed during purification. For example, when membrane-free extract was filtered on Sephadex G-100, only 5% of the ligase activity was recovered (Fig. 1, see also Table III). In order to increase the recovery, protein from the ligase peak was reassayed in the presence of samples from other fractions of this column. This experiment revealed a stimulator peak. When the ligase was assayed in the presence of stimulator, 80 to 90% of the ligase activity was recovered. The n-alanine-activating enzyme, a possible component of the in

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**Table I**

**Requirements for n-alanine incorporation**

The complete reaction mixture contained: 25 mM MgCl₂; 5 mM disodium ATP; 0.04 mM n-[14C]alanine; 1.8 μg of ligase and 13.5 μg of stimulator (Sephadex G-100); 170 μg of membrane fragments; 30 mM piperazine-acetate buffer, pH 6.5, in a final volume of 50 μl. The "boiled fractions" were heated at 100° for 2 min. The amount incorporated was determined by the method described under "Alanine Incorporation Assay."

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity (pmoles/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>92.6</td>
</tr>
<tr>
<td>- Membrane fragments</td>
<td>4.2</td>
</tr>
<tr>
<td>- Stimulator</td>
<td>8.4</td>
</tr>
<tr>
<td>- Ligase</td>
<td>4.6</td>
</tr>
<tr>
<td>- Stimulator and ligase</td>
<td>3.7</td>
</tr>
<tr>
<td>- ATP</td>
<td>3.5</td>
</tr>
<tr>
<td>- Mg²⁺</td>
<td>44.7</td>
</tr>
<tr>
<td>Zero time</td>
<td>1.8</td>
</tr>
<tr>
<td>Boiled membrane fragments</td>
<td>2.3</td>
</tr>
<tr>
<td>Boiled stimulator</td>
<td>9.1</td>
</tr>
<tr>
<td>Boiled ligase</td>
<td>34.8</td>
</tr>
</tbody>
</table>

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![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Resolution of n-alanine:membrane acceptor ligase and stimulator on Sephadex G-100. The supernatant fraction (328,000 X g) (34 mg) was applied to a column of Sephadex G-100 (2.8 X 40 cm). The column was eluted with 5 mM piperazine-chloride, pH 6.0. The fractions were assayed for ligase with the "Alanine Incorporation Assay" (○, -S). Protein (1.8 μg) from the ligase peak (130 ml) was reassayed in the presence of samples from the other column fractions. The results (A) are presented as relative enhancement (stimulation) of the n-alanine incorporation observed with the ligase fraction. In (●, +S), the column fractions were assayed for ligase activity in the presence of 13.5 μg of protein from the stimulator peak (90 ml). The n-alanine-activating enzyme was measured as described under "Experimental Procedures" (■).
Edited range of concentration. In evaluating the properties of
the stimulator and ligase, the concentrations were chosen to
be limiting with respect to the factor under study and saturat-
ing with respect to the second factor.

Nucleotide Specificity—In studies with the unfractionated
extract (Table II), the requirement for ATP could be satisfied
by GTP, CTP, or UTP. It was proposed that the low nucleo-
tide specificity resulted from the presence of excess stimulator.
Thus, a poor nucleotide triphosphate in the presence of n-
alanine and excess stimulator could provide sufficient activated
D-alanine to saturate the incorporation system. As illustrated
in Table II, decreasing the ratio of stimulator to ligase resulted
in an increasing specificity for ATP. For example, at the
lowest ratio, GTP had no activity, CTP effected 3% activity,
and UTP produced 10% of the incorporation activity observed
with ATP. Thus, high specificity for ATP was apparent when
stimulator was present in limiting concentrations with respect
to ligase.

Resolution of Stimulator—In previous work (8), it was not
possible to exclude a role for the D-alanine-activating enzyme
in the incorporation system. This was based on the significant
overlap of the ligase and the D-alanine-activating enzyme in
chromatographic separations. In the present work (Fig. 1),
resolving the ligase from the D-alanine-activating enzyme was accompanied by a large loss in incorporation ac-
tivity which was restored by the addition of stimulator. As
illustrated in Fig. 1, the stimulator co-eluted with the D-alanine-
activating enzyme. Thus, in order to clarify the role of the
stimulator, a resolution of this enzyme from the D-alanine-
activating enzyme was desired. In addition, a complete resolu-
tion of the stimulator and ligase was also desired for further
studies.

The stimulator and ligase, which were partially purified on
Sephadex G-100, were chromatographed on DEAE-cellulose.
The column was equilibrated with 0.02 M piperazine-acetate,
PH 6.0 (0.03 M in acetate), and developed with a linear gradient
of the starting buffer and 0.2 M piperazine-acetate, pH 6.0
(0.3 M in acetate). As illustrated in Fig. 3, A and B, the ligase
was eluted at 0.24 M acetate, and the stimulator was eluted at
0.03 M acetate. The stimulator was also chromatographed on
DEAE-cellulose equilibrated with 0.01 M Tris-HCl, pH 7.8,
containing 0.02 M NaCl. The column was developed with a
linear NaCl-gradient between 0.02 M and 0.20 M NaCl. The
stimulator was recovered at 0.14 M NaCl. On both columns
(Figs. 3B and 4), the D-alanine-activating enzyme co-eluted
with the stimulator. Although complete separation of the
ligase and stimulator was achieved, the D-alanine-activating
enzyme and stimulator could not be resolved on DEAE-cellu-
lose.

A summary of the purification procedure is presented in
Table III. The values in the parentheses (Columns 1 and 2)
represent the specific incorporation activities without added
ligase or stimulator. The values without parentheses represent
specific activities with the second factor at saturating levels
(see “Alanine Incorporation Assay”). The addition of ligase
to the 388,000 × g supernatant fraction resulted in a 3.3-fold
increase in the specific incorporation activity. However, the
addition of stimulator to this supernatant fraction did not in-
crease the specific activity of the incorporation system. These
observations are consistent with an extract that contains excess
stimulator and limiting ligase (see “Nucleotide Specificity”).
Gel filtration and chromatography on DEAE-cellulose resulted
in a 200-fold increase in the specific activity of the ligase. The
purified ligase has an absolute requirement for stimulator.

Table II

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nucleotide</th>
<th>ATP</th>
<th>CTP</th>
<th>CTP</th>
<th>UTP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1. Extract</td>
<td>ATP CTP CTP UTP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>108.4</td>
<td>108.4</td>
<td>113.3</td>
<td></td>
</tr>
<tr>
<td>11.0 Stimulator</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>108.4</td>
<td>108.4</td>
<td>113.3</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>100</td>
<td>108.4</td>
<td>108.4</td>
<td>113.3</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>108.4</td>
<td>108.4</td>
<td>113.3</td>
<td></td>
</tr>
</tbody>
</table>

Citation: J. Biol. Chem. 242, 3193-3198 (1967)
Purification of d-alanine:membrane acceptor ligase and stimulator
The "Alanine Incorporation Assay" and the assay for the d-alanine-activating enzyme were used as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Incorporation</th>
<th>Yield</th>
<th>Specific activity of d-alanine-activating enzyme</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulator</td>
<td>1.0</td>
<td>100%</td>
<td>%</td>
</tr>
<tr>
<td>Ligase</td>
<td>1.2</td>
<td>(100)</td>
<td>100%</td>
</tr>
<tr>
<td>Stimulator</td>
<td>1.2</td>
<td>(100)</td>
<td>100%</td>
</tr>
<tr>
<td>Ligase</td>
<td>100%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1. Supernatant fraction (368,000 x g)</td>
<td>1.2</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>2. Sephadex G-100</td>
<td>35.0</td>
<td>82</td>
<td>52</td>
</tr>
<tr>
<td>3. DEAE-cellulose</td>
<td>230.0</td>
<td>21</td>
<td>10</td>
</tr>
</tbody>
</table>

a Detected in the stimulator fractions.
b Assayed in the absence of added ligase.
c Assayed in the absence of added stimulator.

Purification of the stimulator by these techniques resulted in a 7-fold increase in specific activity and a preparation that has an absolute dependence on ligase.

As previously indicated, the d-alanine-activating enzyme copurified with the stimulator in both fractionations (Figs. 1, 3B, and 4). The increases in specific activity (6) and recovery (10%) were similar to the increases in specific activity (7) and recovery (12%) observed for the stimulator (Table III).

Attempts to Distinguish between Stimulator and d-Alanine-activating Enzyme—Since the chromatographic techniques failed to resolve the stimulator and the d-alanine-activating enzyme, inhibition studies were undertaken in an attempt to differentiate between them. Heat inactivation of the stimulator was performed at 45°, and at the indicated times samples were assayed for stimulator and d-alanine-activating enzyme (Fig. 5A). No significant difference was observed in the time-dependent inactivation of these activities (t1/2 = 3.4 min). In Fig. 5B, the effects of p-hydroxymercuribenzoate on the d-alanine-activating enzyme and stimulator are compared. Samples of the stimulator were preincubated at 25° for 10 min with increasing concentrations of p-HMB. The d-alanine-activating enzyme and the stimulator were both inhibited 50% in the presence of 1.7 μM p-HMB. For comparison, the ligase retained 93% of its activity after preincubation in the presence of 20 μM p-HMB. Thus, it was not possible to distinguish between the stimulator and the activatib enzyme with p-HMB.

The addition of inorganic pyrophosphate to reaction mixtures containing the d-alanine-activating enzyme resulted in a significant inhibition of activity (10). If the activating enzyme is involved in the initial stage of d-alanine incorporation into teichoic acid, the addition of pyrophosphate may also inhibit the incorporation. For these experiments, stimulator which had been purified on DEAE-cellulose was used. This prepara-

The abbreviation used is: p-HMB, p-hydroxymercuribenzoate.
Aspiration is decreased.

velocity remained constant with decreasing stimulator concentration. The "Alanine Incorporation Assay" in the presence of 4.0 µg of ligase. The samples were also assayed for the D-alanine-activating enzyme as described under "Experimental Procedures." In B, samples of stimulator (1.0 mg per ml) in 5 mM Tris-HCl, pH 7.8, were incubated at 25°C for 10 min in the presence of the increasing concentrations of p-HMB. The samples were assayed for stimulator (5.2 µg) with the "Alanine Incorporation Assay" in the presence of 2.0 µg of ligase. The samples were also assayed for the D-alanine-activating enzyme as described under "Experimental Procedures." Fig. 6 (right). Inhibition of alanine incorporation by inorganic pyrophosphate. The "Alanine Incorporation Assay" included 2.6 µg of ligase and 0.8 ( ), 1.4 ( ), and 2.0 ( ) µg of stimulator in the presence of increasing concentrations of pyrophosphate. Increasing concentrations of orthophosphate were also tested in this system with 2.0 µg of stimulator and 2.6 µg of ligase ( ).

FIG. 5 (left and center). Inactivation of the stimulator by heat and by p-hydroxymercuribenzoate. In A, samples of stimulator (1.35 mg per ml) in 5 mM piperazine-chloride, pH 6.0, were maintained at 45°C for the indicated times. The samples were cooled to 4°C and assayed for stimulator (0.7 µg) with the "Alanine Incorporation Assay" in the presence of 4.0 µg of ligase. The samples were also assayed for the D-alanine-activating enzyme as described under "Experimental Procedures." In B, samples of stimulator (1.0 mg per ml) in 5 mM Tris-HCl, pH 7.8, were incubated at 45°C for the indicated times. The samples were cooled to 4°C and assayed for stimulator (6.7 pg) with the "Alanine Incorporation Assay" in the presence of 4.0 pg of ligase ( ).

FIG. 7. Effect of the stimulator concentration on the apparent Kₘ of D-alanine (Lineweaver-Burk plot). The "Alanine Incorporation Assay" was used with 4.0 µg of ligase and the indicated concentration of stimulator: 0.7 µg ( ); 1.8 µg ( ); 11.5 µg ( ). The inset is a plot of the velocity versus increasing substrate concentration.

As illustrated in Fig. 7, decreasing the stimulator concentration resulted in an increase in the amount of D-alanine required to saturate the incorporation system. Although the maximum velocity remained constant with decreasing stimulator concentrations, the apparent Kₘ for D-alanine increased from 18 µM to 2 mM. Thus, this experiment is consistent with the proposal that the D-alanine-activating enzyme functions in the incorporation of D-alanine into membrane teichoic acid.

**DISCUSSION**

A second enzyme, the stimulator, has been identified as a necessary component for the in vitro incorporation of D-alanine into membrane teichoic acid of L. casei. The stimulator and D-alanine:membrane acceptor ligase differ significantly with respect to exclusion volume on Sephadex G-100, heat lability, and sensitivity to p-HMB. In addition, they are completely resolved on DEAE-cellulose.

From our data we suggest that the stimulator may be the D-alanine-activating enzyme. For example, the stimulator and D-alanine-activating enzyme co-elute on Sephadex G-100 and on DEAE-cellulose at pH 6.0 and pH 7.8. The D-alanine incorporation reaction has a high degree of structural and stereochemical specificity for the amino acid (8), a specificity which is essentially identical with that observed for the D-alanine-activating enzyme (10). Both the D-alanine-activating en-
zyme and the stimulator are inhibited to the same extent by preincubation at 45° and by preincubation in the presence of p-HMB. The D-alanine-activating enzyme is characterized by a high specificity for ATP, product inhibition by pyrophosphate, and a high $K_m$ for D-alanine. Under assay conditions in which the stimulator concentration is limiting with respect to ligase, the D-alanine incorporation system, likewise, has a high specificity for ATP and is inhibited by pyrophosphate. As the concentration of stimulator is decreased in relation to the ligase, the apparent $K_m$ for D-alanine increases from 18 μM to 2 μM. This experiment reveals the high $K_m$ of the stimulator for D-alanine, a feature of the D-alanine-activating enzyme. Thus, our results are consistent with the participation of the D-alanine-activating enzyme in the D-alanine incorporation system.

A two-stage reaction sequence for the incorporation of D-alanine into membrane teichoic acid is suggested. In Reaction 1, D-alanine is activated by the D-alanine-activating enzyme in the presence of ATP to form an enzyme·AMP·D-alanine complex with the release of inorganic pyrophosphate. In Reaction 2, the activated D-alanine is transferred to membrane teichoic acid in the presence of ligase.

The precise role of the D-alanine:membrane acceptor ligase in Reaction 2 is open to speculation. For example, ligase may facilitate the binding of the activated D-alanine enzyme complex with a membrane site prior to alanine incorporation. This role would be analogous to that of nectin, a protein necessary for the binding of ATPase to the membranes of Streptococcus faecalis (16). Alternatively, the ligase may catalyze the transfer of activated D-alanine to the membrane teichoic acid.

In the initial studies on the D-alanine-activating enzyme, no explanation could be offered for the high $K_m$ of D-alanine. In this coupled system, however, the important parameter may not be the $K_m$, per se, but rather may be a high rate of activation or an excess of activating enzyme in relation to the rate-limiting step. These factors will ensure that the incorporation system is saturated with activated D-alanine at low substrate concentrations.

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Biosynthesis of Membrane Teichoic Acid: A ROLE FOR THE
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