Biogenesis of Membrane Lipids: Mutants of Escherichia coli with Temperature-Sensitive Phosphatidylserine Decarboxylase*  
(membrane function/phosphatidylethanolamine/psd mutants/phospholipid)

EDWARD HAWROT AND EUGENE P. KENNEDY

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT Phosphatidylserine decarboxylase catalyzes the last step in the pathway leading to phosphatidylethanolamine, the principal membrane lipid of E. coli. Mutants of E. coli have now been isolated in which this enzyme is thermolabile. The structural gene for phosphatidylserine decarboxylase (psd gene) is closely linked to the purA locus at about 83 min on the standard map of the E. coli chromosome. When a mutant with thermolabile decarboxylase is incubated at 42°C, growth ceases, but only after a substantial fraction (20–40%) of the total phospholipid of the cell has been replaced by phosphatidylserine. Examination of such mutants with altered content of phospholipids may shed light on the role of specific phospholipids in membrane function.

Phospholipids are major structural components of biological membranes throughout nature and are also thought to play important functional roles in certain membrane-localized processes. One approach to the study of specific lipid functions is to isolate mutants defective in a single step of the biogenesis of membrane lipids, thus giving rise to cells with a specifically altered pattern of membrane lipids.

Various classes of bacterial mutants deficient in some phase of lipid synthesis have been isolated and have proven useful in studying certain aspects of the metabolism and function of phospholipids (for reviews see refs. 1 and 2). Until recently, the only phospholipid mutants available were affected early in the pathway at the level of phosphatic acid synthesis (3–7). Reports have recently appeared, however, describing the isolation of mutants of Escherichia coli defective in some early stage of the formation of phosphatidylethanolamine, the predominant phospholipid in E. coli (8–10).

This report describes the isolation of mutants with temperature-sensitive phosphatidylserine decarboxylase, the mapping of the structural gene for the decarboxylase (psd gene), and a preliminary characterization of the phenotype of some of these mutants.

MATERIALS AND METHODS

Bacterial and Phage Strains. E. coli strains (described in Table 1) used in this study are all K-12 derivatives. Phage P1c was used for generalized transduction. Phage P1CMdrl00 (11) was obtained from Dr. A. L. Taylor. Transduction was performed essentially according to the method of Lennox (12).

Preparation of Extracts. Single colony isolates were grown in 5 ml of L-broth (13) at 30°C to a density of 5 to 9 × 10^8 cells per ml. The culture was centrifuged at 3500 × g for 30 min and the cells were immediately suspended in 2 ml of a solution of lysozyme (100 µg/ml) (Worthington, 11,800 units/mg) in 25 mM Tris-HCl buffer (pH 8.2) containing 2.5 mM Na2EDTA at 4°C. After approximately 10 min at room temperature the suspensions visibly cleared and the lysates were stored at 4°C until the time of assay.

Assay for Phosphatidylserine Decarboxylase. The assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.2 mM phosphatidyl(1-14)C]serine (10,000 cpm/µmol), and enzyme in a final volume of 0.5 ml. Normally the enzyme extract was incubated in the assay mixture in the absence of the substrate for 30 min at the appropriate temperature and the reaction was started by the addition of 0.1 ml of 1 M phosphatidyl(1-14)C]serine. After 20 min, 0.5 ml of 1 N H2SO4 was added and the amount of radioactive CO2 liberated was determined (14). One unit of activity is the amount of enzyme which forms 1 nmol of product per min at 25°C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A324</td>
<td>F−, lacI, pro, thi, strA</td>
<td>S. Luria</td>
</tr>
<tr>
<td>EH36</td>
<td>psd-I, contains a second, unidentified mutation that prevents growth at 42°C</td>
<td>Derived from A324 after mutagenesis</td>
</tr>
<tr>
<td>EH102</td>
<td>psd-I, normal growth at 42°C</td>
<td>Transductant of EH36 from A324</td>
</tr>
<tr>
<td>EH103</td>
<td>Same as EH102</td>
<td>Same as EH102</td>
</tr>
<tr>
<td>KG20</td>
<td>F+, purA, ampA1, his, argH, pro, thi, strA</td>
<td>K. G. Eriksson-Grenberg</td>
</tr>
<tr>
<td>ES4</td>
<td>F−, purA</td>
<td>CGSC*</td>
</tr>
<tr>
<td>KLF17/KL132 F117/ppyB, thi, pro, his, recA, thy, rel, thi, leu, strA</td>
<td>CGSC*</td>
<td></td>
</tr>
<tr>
<td>EH140</td>
<td>F117 purA+/ES4 purA (P1CMdrl00 lysogen)</td>
<td>Episome transfer; KLF17/KL132 × ES4, followed by infection with P1CMdrl00</td>
</tr>
<tr>
<td>EH150</td>
<td>psd-B(t), purA +</td>
<td>Transductant of ES4 (see text)</td>
</tr>
<tr>
<td>EH180</td>
<td>Revertant of EH150 that grows normally at 42°C</td>
<td>Spontaneous revertant</td>
</tr>
</tbody>
</table>

* The work described here forms part of a dissertation to be submitted to the Faculty of Arts and Sciences of Harvard University in partial fulfillment of the requirements for the Ph.D. degree.

* E. coli Genetic Stock Center, Yale University, New Haven, Conn.
Fig. 1. Temperature profile of phosphatidylserine decarboxylase activity in extracts of E. coli A324 and EH36. Bacteria were grown at 30° to 5 to 6 × 10^6 cells per ml in L-broth. Extracts were incubated for 12 min in the assay mixture in the absence of the substrate (phosphatidyl[1-14C]serine) at the temperatures indicated. Substrate was then added, and decarboxylase activity was measured as the release of 14CO2 during 20 min further incubation at the same temperature. (O) A324 extract; (Δ) EH36 extract.

Materials. DL-[1-14C]serine, DL-[3-2H]serine, and [32P]phosphoric acid were products of the New England Nuclear Co., Boston. Phosphatidyl[1-14C]serine was prepared enzymatically as previously described (15).

RESULTS

On the assumption that the phosphatidylserine decarboxylase is essential for the continued growth of E. coli, we examined a number of conditional lethal mutants, unable to grow at 42°, for a temperature-sensitive decarboxylase. One such strain (EH36) was indeed found to have an altered decarboxylase. However, this alteration was not responsible for the observed temperature-sensitivity of growth, since the mutant strain did not accumulate phosphatidylserine in vivo. EH36 is, therefore, a double mutant, with a lesion in the structural (psd) gene for the decarboxylase, and a mutation, as yet unidentified, responsible for the inability to grow at 42°. The mapping of the psd mutation, however, made it possible to select other mutants, in which phosphatidylserine accumulates in vivo at 42°, preventing growth at that temperature.

Isolation of Strain EH36. Mutagenesis was performed on A324 cells according to the method of Adelberg et al. (16) with 300 μg/ml of N-methyl-N′-nitro-N-nitrosoguanidine in Tris-maleate buffer (pH 6.0) containing 0.2% glucose for 30 min at 37°. Approximately 2% of the survivors, plated out on L-broth agar at 30°, were unable to grow on similar plates at 42°, as revealed by replica plating. Of 92 temperature-sensitive isolates examined, one (EH36 psd-1) had less than 1% of wild-type phosphatidylserine decarboxylase activity in extracts at 42°.

As shown in Fig. 1, an extract prepared from EH36 cells has an altered temperature profile with near normal levels of activity at the low temperatures. Extracts of the mutant did not inhibit the decarboxylase activity observed in extracts of A324 at 42°, arguing against the existence of a heat-activated inhibitor or phospholipase.

Characterization of Altered Phosphatidylserine Decarboxylase in the psd-1 Mutant. Recombinants of EH36 were obtained by P1 transduction, which were able to grow at 42°, but which nevertheless exhibited the same thermolabile decarboxylase activity observed in EH36. Clearly the altered decarboxylase is not the reason for the failure of EH36 to grow at 42°.

The thermal lability of the mutant decarboxylase was examined in one such psd-1 recombinant in the experiment of Fig. 2. The mutant enzyme is rapidly inactivated by treatment at 40° in the presence of 0.1% Triton X-100, but not in its absence. The same concentration of Triton X-100 has little or no effect on the wild-type enzyme. However, at a higher concentration of Triton (1.1%) even the wild-type enzyme is inactivated at 40°, as shown in Fig. 3. Thus, as a result of the psd-1 mutation, the enzyme has acquired a higher sensitivity to the combined treatment with detergent and heat. The mutant enzyme is protected to a considerable degree against

Fig. 2. Thermolability of the phosphatidylserine decarboxylase activities in extracts of strains A324 and EH103. Sonicated extracts of A324 and EH103 cells grown at 37° were incubated in phosphate buffer in the presence or absence of 0.1% Triton X-100 at 40°. At the indicated times, samples were removed and their initial rates of decarboxylase activity were determined at 25°. The data are plotted relative to unheated samples ( = 100%). (O) A324 extract + 0.1% Triton; (Δ) A324 extract, no Triton; (O) EH103 extract + 0.1% Triton; (Δ) EH103 extract, no Triton.

Fig. 3. Triton-sensitivity of phosphatidylserine decarboxylase activities in extracts of A324 and EH102 at 40°. Triton-extracted enzyme fractions (Fraction 2, Table 2) of A324 and EH102 cells were incubated in phosphate buffer at two different Triton concentrations (0.1% and 1.1%) at 40°. One sample of EH102 extract was in addition incubated in the presence of both 0.1% Triton and 20% glycerol at 40°. At the indicated time intervals, samples were removed and their initial rates of decarboxylase activity were determined at 25°. The data are plotted relative to unheated samples ( = 100%). (O) A324 extract + 0.1% Triton; (Δ) A324 + 1.1% Triton; (■) EH102 extract + 0.1% Triton; (O) EH102 extract + 1.1% Triton; (■) EH102 extract + 0.1% Triton + 20% glycerol.
Fig. 4. DEAE-cellulose chromatography of a combined extract of A324 and EH102 cells. Enzyme extracts of A324 and EH102 cells (Fraction 3, Table 2) were mixed to produce a ratio of mutant to wild-type enzyme of 3:1. This combined extract was applied to a DEAE-cellulose column (1.3 cm × 12 cm; Whatman DE-52) at 25°C in 10 mM potassium phosphate buffer (pH 7.4) containing 1% Triton X-100, 10% glycerol, and 10 mM 2-mercaptoethanol. After the column was washed with this buffer, the enzyme was eluted with a linear gradient of NaCl in the same buffer. Decarboxylase activity was determined at 25°C and at 42°C as described in Materials and Methods. Both peaks of activity were eluted at 0.18 M NaCl. (●) Molarity of NaCl; (○) phosphatidylserine decarboxylase activity at 25°C; (△) phosphatidylserine decarboxylase activity at 42°C.

Purification of the Decarboxylase from a psd-1 Strain. The purification of the thermosensitive decarboxylase was found to be identical with that of the wild-type enzyme through at least the first four steps (Table 2 and Fig. 4). At each stage the mutant decarboxylase retained its characteristic thermosensitive behavior, exhibiting less than 1% of wild-type activity at 42°C under the standard assay conditions. When preparations of wild-type and mutant enzyme were mixed and chromatographed, the peak of the thermosensitive activity was eluted in the same fractions as wild-type decarboxylase (Fig. 4). The psd-1 mutation thus leads to the production of a slightly altered enzyme with reduced thermal stability in this combined treatment by the presence of 20% (v/v) glycerol (Fig. 3).

Table 2. Purification of mutant and wild-type phosphatidylserine decarboxylase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A324</th>
<th>EH102</th>
<th>A324</th>
<th>EH102</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell-free extract</td>
<td>18</td>
<td>10</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>2. Triton extract</td>
<td>48</td>
<td>32</td>
<td>76</td>
<td>69</td>
</tr>
<tr>
<td>3. Acetone treatment</td>
<td>61</td>
<td>39</td>
<td>57</td>
<td>45</td>
</tr>
</tbody>
</table>

A324 and EH102 psd-1 cells were grown and phosphatidylserine decarboxylase was purified essentially as described previously (14).

Table 3. Failure of EH102 psd-1 to accumulate phosphatidylserine in vivo

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incorporation of DL-[1-14C]serine into lipid (cpm/ml of culture)</th>
<th>Incorporation of DL-[3-3H]serine into lipid (cpm/ml of culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A324 psd+</td>
<td>457</td>
<td>288</td>
</tr>
<tr>
<td>EH102 psd-1</td>
<td>289</td>
<td>330</td>
</tr>
</tbody>
</table>

Bacterial cultures (6 ml) were grown in minimal medium at 30°C to a cell density of about 5 × 10⁹/ml. At this point half of each culture was shifted to 42°C. After 15 min further incubation DL-[1-14C]serine and DL-[3-3H]serine was added at a final concentration of 0.05 mM. The specific activity of the DL-[1-14C]serine was 5 × 10⁶ cpm/μmol and that of the DL-[3-3H]serine was 10⁶ cpm/μmol. Labeling was terminated after 30 min by the addition of an equal volume of 1 N HCl and the immediate chilling of the suspension. Carrier cells were added and the suspensions were centrifuged at 8000 × g for 30 min at 4°C. The pellets were extracted with 3 ml of methanol for 15 min at 55°C. After the extracts were cooled to room temperature, chloroform (6 ml) was added with thorough mixing. The lipid phase was washed twice with 10 ml of distilled water. A portion (2 ml) of the chloroform layer was dried and its radioactivity was measured.

The presence of detergent, but with other properties closely similar to those of wild-type enzyme.

Activity of Phosphatidylserine Decarboxylase In Vivo in the psd-1 Mutant. Strain EH102, bearing the psd-1 mutation, grows at 42°C at the same rate as wild-type. The experiments of Figs. 2 and 3 explain this finding, since the membrane-bound mutant enzyme is almost as stable as wild-type enzyme, and its altered stability is detected only in the presence of added detergent.

Phosphatidylserine is present only in trace amounts in normal cells of E. coli (15), since it is decarboxylated almost as rapidly as it is formed. To test the function of the decarboxylase in the psd-1 mutant in vivo, mutant EH102 and control parental cells were labeled with a mixture of [1-14C]-serine and [3-3H]serine at 30°C and at 42°C (Table 3).

The incorporation of [3-3H]serine into phospholipid should reflect the amount of synthesis of phosphatidylethanolamine plus phosphatidylserine. The incorporation of [1-14C]serine

Table 4. Three-factor cross between psd, purA, and ampA markers

<table>
<thead>
<tr>
<th>Donor Strain</th>
<th>Recipient Strain</th>
<th>Selection Marker</th>
<th>Type</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EH102 (psd-1)</td>
<td>KG20 (psd+, Pur+)</td>
<td>ampA1, psd+ 12</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ampA+, purA+</td>
<td>ampA1, purA</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

A P1 lysate of strain EH102 psd-1 was prepared and used for generalized transduction with KG20 ampA1, purA as recipient. Selection was for Pur+ recombinants, which occurred with a frequency of approximately 40/10⁵ plaque-forming units. Twenty-nine recombinants were isolated, tested for ampicillin sensitivity, and assayed for thermolabile phosphatidylserine decarboxylase activity.
should in turn reflect the amount of accumulated phosphatidylserine, since 1-\textsuperscript{14}C-radioactivity will be lost upon decarboxylation to phosphatidylethanolamine. Only traces of phosphatidylserine accumulate in EH102 or in wild type, at either 30° or 42° (Table 3).

**Genetic Mapping of the psd Locus.** Preliminary mapping of the psd-1 mutation in EH36 was accomplished by standard conjugation mapping techniques (to be described in detail elsewhere). Final localization was achieved by transduction with phage P1. The psd-1 locus is 59–72% cotransducible with purA and approximately 6% cotransducible with melA. No cotransduction was observed with pyrB. The psd locus is thus placed at about 83 min on the Taylor and Trotter map (17). The gene order in this region was determined by a triple cross performed again by P1 transduction. As shown in Table 4, the least frequent recombinant class is purA\textsuperscript{+}, ampA\textsuperscript{+}, psd\textsuperscript{+}, and if this is presumed to require a quadruple cross-over, the gene order in this region is ampA-psd-purA.

**Isolation of Conditional Lethal Phosphatidylserine Decarboxylase Mutants.** To obtain decarboxylase mutants that are expressed in vivo, as well as in vitro, a strategy was developed, using a variation on the theme of localized mutagenesis (18). It should be generally applicable for any genetic locus with a known high-frequency cotransducing marker. Strain EH140 (Table 1) contains the P117 episome (19) with the psd\textsuperscript{+} gene closely linked to the purA\textsuperscript{+} locus. Another copy of the psd\textsuperscript{+} gene lies on the chromosome; thus a temperature-sensitive mutation in the episomal psd gene should not prevent the growth of the strain at higher temperatures. It is also lysogenic for phage P1CMdr100 (11) so that high titer lysates can easily be produced.

This strain was subjected to mutagenesis with N-methyl-N'\textsuperscript{-}nitro-N\textsuperscript{-}nitrosoguanidine. A P1 lysate was prepared (13) and used for the transduction of the episomal purA\textsuperscript{+} gene with its closely linked psd locus to an unmutagenized purA recipient (ES4). The purA\textsuperscript{+} recombinants, grown out at 30°, were tested for growth at 42° by replica-plating. Temperature-sensitive mutants thus isolated should result from mutations cotransduced with the purA\textsuperscript{+} gene. From the cotransduction frequency of psd and purA, it is apparent that about 60% of the purA\textsuperscript{+} recombinants must also have received their psd genes via P1 transduction from the mutagenized EH140 cells. Thus, some of the temperature-sensitive mutants should be caused by mutations in the psd locus.

Of approximately 45,000 purA\textsuperscript{+} recombinants examined, 11 were unable to grow on L-broth plates at 42°, and were shown not to be lysogenic for phage P1CMdr100. Of the 11, three have a defective phosphatidylserine decarboxylase as measured under standard assay conditions. In addition, all three of these mutants accumulate phosphatidylserine in vivo at 42° and are thus of the phenotype sought for studies of membrane function.

One such mutant (EH150 psd-2\textsubscript{(ts)}), was compared with a revertant, EH180, with recovered ability to grow at 42°. The lipid composition of the two strains was measured in the experiment of Fig. 5. Only traces of phosphatidylserine were found in either strain at 30°. However, 4 hr after the shift to 42° (approximately three doubling times) 25% of the phospholipid of the mutant EH150 was in the form of phosphatidylserine, whereas there was no accumulation of this lipid in the revertant EH180. In other experiments with all three mutants, accumulation of phosphatidylserine has ranged from 20 to 40%.

The mutant enzyme in EH150 psd-2\textsubscript{(ts)} is very labile, since no activity can be detected under standard assay conditions even at 30°. However, when 20% glycerol or 20% sucrose was added to the assay mixture, about 20% of wild-type activity could be detected at 30° in the mutant extracts. This activity was completely abolished by prior heating of the extracts at 42°. Thus, the mutant decarboxylase of EH150 can be shown to be thermolabile in vivo, as well as in vitro.

Only a few revertants of EH150 psd-2\textsubscript{(ts)} with restored ability to grow at 42° have so far been examined. All of these contain a decarboxylase apparently more thermostable than that of EH150 psd-2\textsubscript{(ts)}, but less than that of wild-type cells. Extracts of these revertants are similar to extracts of the psd-1 mutant; that is, the decarboxylase is active at 30° under standard assay conditions, but not at 42°. Reversion of these strains thus appears to be caused by intragenic suppression rather than true back-reversion. Like EH36 psd-1, the revertants do not accumulate phosphatidylserine at 42° (as shown in Fig. 5).

**DISCUSSION**

The altered properties of the phosphatidylserine decarboxylase caused by the psd-1 mutation offer a striking illustration of
the stabilization of an enzyme by its association with the cell membrane. While membrane-bound, the mutant enzyme is little affected by heating at 40°, but in the presence of the detergent Triton X-100 it is completely inactivated. It is possible that many potentially lethal mutations in membrane-bound enzymes may be phenotypically silenced by the unique stabilizing environment of the membrane. This may be especially significant in the search for temperature-sensitive mutants, since hydrophobic interactions are strengthened at higher temperatures.

The decarboxylase from the pad-I mutant is active at 30° under standard assay conditions in the presence of Triton X-100. The pad-D(14) mutation leads to the production of an enzyme that is inactivated at 42° in vitro and displays no activity in vivo in the presence of Triton even at 30°; it is thus much less stable than the enzyme produced in the pad-I mutant. Significantly, the limited number of temperature-resistant revertants so far studied are similar to the pad-I mutant in that the revertant enzymes are apparently less thermostable than true wild-type decarboxylase. Either the pad-D(14) mutant, or these revertants, may represent double-mutant alleles of the wild-type gene.

The isolation of conditional lethal mutants, unable to grow at a temperature at which the phosphatidylserine decarboxylase does not function in vitro, confirms the initial assumption of the essential nature of the decarboxylase to the continued growth of E. coli and supports the proposed metabolic pathway for the synthesis of phosphatidylethanolamine in this organism (15). Cessation of growth in these mutants could result from the lack of sufficient phosphatidylethanolamine to participate in some vital membrane function for which other phospholipid species cannot substitute. On the other hand, the accumulation of phosphatidylethanolamine may itself adversely affect membrane function and prove lethal to the cell. The resolution of such questions obviously requires a detailed study of membrane function under conditions in which the decarboxylase is inoperative.

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12. Lennox, E. S. (1955) "Transduction of linked genetic characters of the host by bacteriophage P1," Virology 1, 190-206.