ISOLATION AND CHARACTERIZATION OF A TEMPERATURE-SENSITIVE MUTANT OF ESCHERICHIA COLI WITH A LESION IN THE ACYLATION OF LYSOPHOSPHATIDIC ACID

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SUMMARY: A temperature-sensitive mutant of E. coli with a defect in acyl-CoA: monoacylglycerol 3-phosphate acyltransferase has been isolated. The acyl-CoA: glycerophosphate acyltransferase and CTP:phosphatidic acid cytidyltransferase of this mutant behaved normally on heating. Analysis of the phospholipid fatty acids from the mutant grown at or below 35C showed an abnormally high ratio of unsaturated plus cyclopropane to saturated fatty acids. The synthesis of phospholipids, RNA, and DNA was rapidly shut off in the mutant exposed to 42C, while protein synthesis continued for at least one hour. After one hour at 42C more than 80% of the cells died rapidly.

The isolation of bacterial mutants with defective phospholipid synthesis should lead to a better understanding of the mechanisms of formation and the genetic control of these major membrane components. Since no mutants with requirements for preformed phospholipids have been isolated, we have attempted to isolate conditional mutants with temperature-sensitive phospholipid synthesis.

Phospholipid synthesis in E. coli starts with a two step transacylation between fatty acyl thiolesters and glycerol 3-phosphate to form sequentially monoacyl glycerol 3-phosphate (lysophosphatidic acid or LPA) and phosphatidic acid (PA); the latter in turn reacts with CTP to form CDP-diglyceride, a central intermediate in phospholipid biosynthesis (1). Cronan and Vagelos (2) have recently reported the isolation of a temperature-sensitive mutant of E. coli defective in the first transacylation. We wish to report the isolation and characterization of a temperature-sensitive mutant with a lesion in the second transacylation reaction.

METHODS:

Isolation of Mutants: 500 ts⁻ mutants of E. coli CR34, a derivative of E. coli K12, were obtained by ethyl methane sulfonate mutagenesis (3). Potential phospholipid mutants were picked on the basis of their incorporation of ^{32}P-orthophosphate into phosphatidyl ethanolamine (PE), phosphatidyl glycerol plus cardiolipin (PG) and nucleic acids during 90 min of incubation of the mutants at 42C in trypticase soya broth enriched with 10 μg/ml of thymine (TSB-T). These measurements were made after direct chromatography of TCA-precipitated cells on anion exchange paper according to the method of Shibuya and Maruo (4). Mutants were further screened by a study of the kinetics of incorporation of $^{32}P_i$ in PE, PG, and nucleic acids at 42C in order to eliminate mutants in which phospholipid synthesis did not become completely blocked. Six mutants were selected, of these GH352 was studied further.

Preparation of Enzymes: The particulate enzymes for the transacylase reactions were prepared from cells grown in TSB-T to 180 Klett$_{54}$ units at 30C. The cells were centrifuged and washed in 77 mM phosphate buffer pH 7 with 0.92 mM 2-mercaptoethanol. They were then suspended in the same buffer and sonicated. After centrifugation at 5090 xg for 10 min at 4C, the supernatant solution was recentrifuged at 90,000 xg for 30 min. The pellets, which contained the transacylases, were either suspended in the phosphate-mercaptoethanol buffer for the thin-layer chromatographic (TLC) assay (5) or in phosphate buffer for the spectrophotometric assay (6). LPA was mainly 1-palmityl glycerol 3-phosphate.

RESULTS:

Upon exposure to 42C, strain GH352 showed a 50% increase in viable count in the first half hr. Little change was observed during the next half hr followed by a steep decrease in viability. Although greater than 80% of the cells were killed at 42C, there was little lysis as evidenced by the small decrease in turbidity (Fig. 1). The cells which were viable after 5 hr at 42C were still unable to grow at 42C.

Pulse-labelling experiments showed that the rate of ^{14}C-leucine incorporation

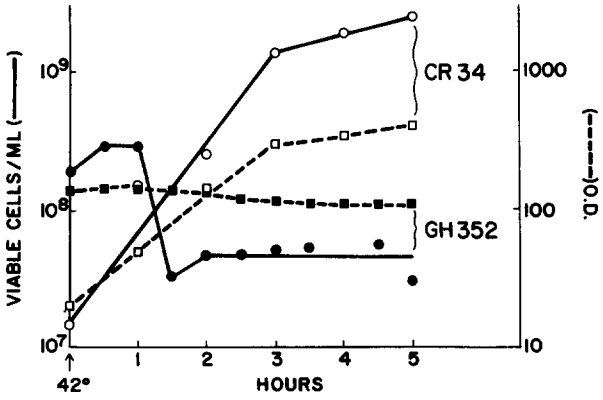

Fig. 1. Growth of CR34 and GH352 at 42C. The cells were grown at 30C for 2 hr prior to the temperature shift.

into protein doubled during the first 20 min at 42C and then decreased to the normal level at 60 min. The decrease continued to 15% of normal at 100 min. The rates of $^{32}P_i$ incorporation into PE, PG, and nucleic acids decreased steadily at 42C (Fig. 2). Pulse labelling with ^{3}H-thymine and ^{3}H-uracil showed that both DNA and RNA synthesis were affected.

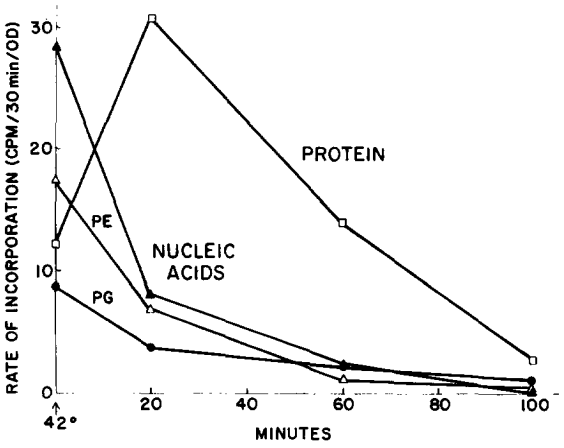

Fig. 2. Protein, phospholipid, and nucleic acid synthesis at 42C. Pulse labelling experiments. Cells were grown in TSB-T and either $^{32}P_i$, 6 μc/ml or ^{14}C-leucine, 2 μc/ml was added at various times after the temperature-shift. Phospholipid and nucleic acid synthesis were measured as described in the text. A filter paper disc assay was used for ^{14}C-leucine incorporation (7). Rate of: ^{14}C-leucine incorporation □——□ ; Rate of $^{32}P_i$ incorporation into: Nucleic acids ▲——▲; phosphatidyl ethanolamine Δ——Δ; Phosphatidyl glycerol plus cardiolipin ●——●.

Since both branches of the phospholipid pathway were affected at 42C, the thermolability of the enzymes before the branch was investigated. A TLC study of the kinetics of glycerol 3-phosphate acylation by particles from both CR34 and GH352 showed that label appeared in LPA first, a steady rate of biosynthesis being reached in less than 1 min, and that accumulation of label in LPA leveled off at 8 to 10 min. PA biosynthesis showed a distinct lag followed by a linear phase. Particles from both CR34 and GH352, preincubated at 42C for 2 hr, accumulated ^{14}C in LPA for 15 to 20 min, and synthesized LPA at a rate about 2-fold higher than that seen in untreated particles.

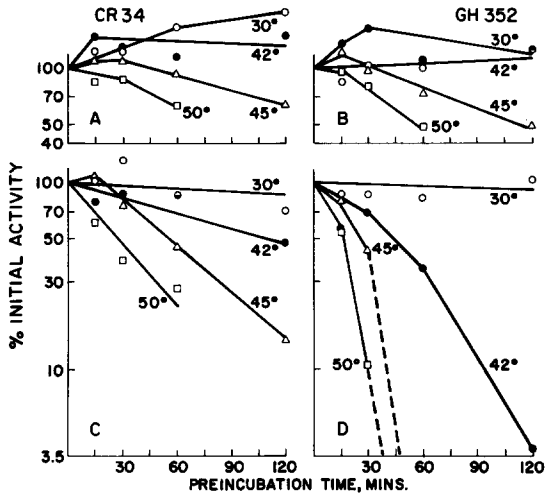

Fig. 3. Thermolability of glycerophosphate and LPA acylation. Preincubation mixture (vol 75 µl): KPO_4, pH 7.0, 193 mM; $MgCl_2$, 13.3 mM; particles 0.125 mg protein. Reaction at 24C (vol 200 µl) contained the above plus; glycerol 3-phosphate, 10 mM or LPA, 0.5 mM; 5,5'-Dithiobis-2-nitrobenzoate, 1 mM; palmityl CoA, 0.05 mM; bovine serum albumen, fatty acid free, 200 µg. The reaction was measured at 413 nm in a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbance recorder. A and B, glycerol 3-phosphate acylation; C and D, LPA acylation. A and C, CR34; B and D, GH352. The dashed lines indicate that there was no measurable activity at the next preincubation time.

However, the rate of PA formation was diminished by 15% in CR34 and 65% in GH352, when preincubated particles were compared with untreated particles.

The rate of transacylation as measured by a spectrophotometric assay (Fig. 3) showed that glycerol 3-phosphate acylation was essentially unaffected in both the mutant and parent strains after 2 hr preincubation at 30 and 42C. Preincubations at 45C and 50C resulted in the same kinetics of thermal inactivation in both strains. The kinetics of thermal inactivation of LPA acylation in the mutant strain were clearly different from that seen in the parent strain, in confirmation of the results obtained with the TLC assay. After 2 hr preincubation at 42C, the percent initial activity for PA biosynthesis from LPA was 5% in the mutant and 47% in the parent strain. On preincubation at 45C, LPA acylation was completely inactivated in the mutant particles after 60 min, while in the parent particles there was 45% of the initial activity. Similar results were obtained with

oleyl-CoA as acyl donor, although the rate of thermal inactivation of the acyltransferase to lysophosphatidic acid was not as rapid as it was with palmityl-CoA as acyl donor, in both the parent and mutant strains. Experiments with mixed extracts showed that the loss of activity on heating the mutant particle was not caused by the formation of an inhibitor.

The kinetics of thermal inactivation for CTP:PA cytidyl transferase (Fig. 4) were the same for both parent and mutant particles on preincubation at 42C and 50C. This excludes the possibility of a temperature-sensitive lesion at this step of phospholipid biosynthesis.

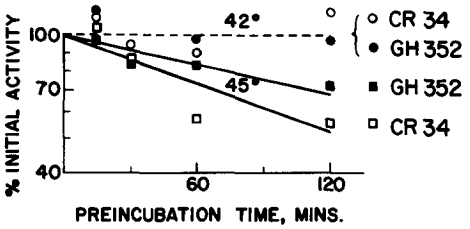

Fig. 4. Thermolability of CTP: phosphatidic acid cytidyl transferase. Preincubation mixture (vol 50 µl): KPO_4, pH 6.5, 0.4 M; enzyme, 0.2 mg protein. Reaction at 30C (vol 200 µl) contained the above plus; phosphatidic acid 2 mM; ^3H-CTP, 1.75 mM, 7.14 µc/µmole. Particles were prepared and assayed as described by Carter (1). The incorporation of ^3H-CTP into lipid was measured by a filter paper disc method, modified by using Whatman #1 filter paper and washing with cold 5% trichloroacetic acid containing 1% $Na_4H_2PO_7$ (2).

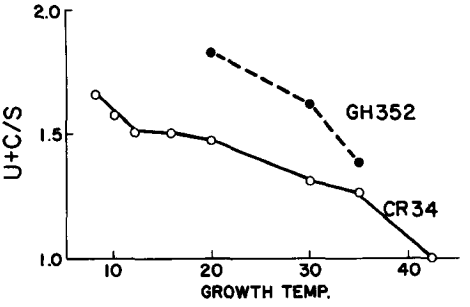

Fig. 5. Ratio of unsaturated plus cyclopropane to saturated fatty acids (U + C/S) in CR34 and GH352 phospholipids. Phospholipids were extracted with chloroform-methanol, separated from non-polar lipids on silicic acid columns, and saponified. The fatty acids were methylated with diazomethane and subjected to gas-liquid chromatographic analysis at 180C on a 10% EGSS-X on gas-chrom P 100/200 mesh column, 1/8 in by 6 ft.

Analysis of the fatty acids of the phospholipids in the parent and mutant strains showed that the mutant had a markedly higher ratio of unsaturated plus cyclopropane fatty acids to saturated fatty acids than the parent strain when the cells were grown at 20C and 30C (Fig. 5). These differences reflect an increase in the proportions of 16:1 + 17:cyclo fatty acids and a decrease in saturated fatty acids in the mutant strain.

DISCUSSION:

Although the results with both the TLC and spectrophotometric assays point to a specific lesion in the acylation of lysophosphatidic acid in GH352, there were some quantitative differences in the kinetics of thermal inactivation as measured by the two assays. Preincubation at 42C resulted in a more rapid inactivation when acylation was measured by the spectrophotometric assay. This difference is attributable to the absence of 2-mercaptoethanol during preincubation prior to the spectrophotometric assay, in which the presence of sulfhydryl compounds is precluded. Similarly, a greater stimulation of glycerophosphate acylation was seen in particles from both parent and mutant cells pre-incubated in the presence of mercaptoethanol than in its absence.

We can offer no definitive explanation for the finding that both DNA and RNA synthesis were also rapidly turned off at 42C. Similar results were not noted in the acyl-transferase I mutant recently described by Cronan and Vagelos (2). One possibility is that there are additional temperature-sensitive enzymes in the mutant we have described. This seems unlikely since a study of three spontaneous revertants showed that reversion to growth at 42C, and correction of the defect in the second acylation appear to occur as a single spontaneous genetic change. The possibility exists that macromolecular synthesis becomes temperature-sensitive at 42C as a result of the high levels of unsaturated and cyclopropane fatty acids present in the phospholipids of strain GH352 grown at 30C. The extra fluidity of the membrane may result in excessive leakage of small molecules when the cells are shifted to 42C. Membrane transport may also be affected (8,9). These hypotheses are currently being tested. Protein synthesis does continue for over 1 hr after a shift to 42C,

and the synthesis of ATP is as rapid in GH352 as it is in the parent strain for 1 hr at 42C. It should also be noted that at 1 hr, when phospholipid, DNA, and RNA syntheses have essentially ceased in strain GH352 and protein synthesis is still nearly normal, over 80% of the cells rapidly die, perhaps as a result of this imbalance in macromolecular synthesis.

Isolation of this mutant, in addition to the mutant described by Cronan and Vagelos (2), lends support to the hypothesis that the additions of two fatty acids to glycerol 3-phosphate are catalyzed by different enzymes (10).

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