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Properties and Biosynthesis of Cyclopropane Fatty Acids in *Escherichia coli*

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The lipid phase transition of *Escherichia coli* phospholipids containing cyclopropane fatty acids was compared with the otherwise homologous phospholipids lacking cyclopropane fatty acids. The phase transitions (determined by scanning calorimetry) of the two preparations were essentially identical. Infection of *E. coli* with phage T3 inhibited cyclopropane fatty acid formation over 98%, whereas infection with mutants which lack the phage coded *S*-adenosylmethionine cleavage enzyme had no effect on cyclopropane fatty acid synthesis. These data indicate that *S*-adenosylmethionine is the methylene donor in cyclopropane fatty acid synthesis.

Although cyclopropane fatty acids (CFA) are a major component of the membrane phospholipids of a large variety of bacteria (11), the function of these acids is unclear. The phospholipids of *Escherichia coli* contain two CFA homologs, *cis*-methylene hexadecanoic acid and *cis*-methylene octadecanoic (lactobacillic) acid (8). These acids are believed to be formed by methyleneation of the unsaturated moieties of the membrane phospholipids by *S*-adenosyl-L-methionine (SAM) in a reaction catalyzed by the enzyme, CFA synthetase (16). We previously isolated mutants deficient in CFA synthetase and in CFA synthesis (25). However, we have not yet detected a physiological phenotype for these mutants and thus have no rationale for the energetically expensive formation of CFA (25).

The physical properties of CFA in the free acid form differ from those of the homologous unsaturated fatty acids (2, 9). These differences led to the proposal that the conversion of phospholipid unsaturated fatty acid moieties to their cyclopropane derivatives could increase the temperature of the order to disorder transition of the membrane phospholipids (13, 18). A large alteration in the transition temperature would have a very significant effect on membrane function (5, 6). However, to our knowledge, no direct measurement of the order to disorder transition of CFA-containing phospholipids has been reported. In this paper we report the order to disorder transition of *E. coli* membrane phospholipids that contain CFA and compare the transition of these lipids with *E. coli* phospholipids which lack CFA.

The control of CFA synthesis of *E. coli* is not understood. CFA synthetase is present throughout the growth cycle of *E. coli*, although the bulk of CFA formation occurs during the transition from log phase to stationary phase (4, 16). Enzymatic studies indicate that the donor of the new methylene carbon of the CFA is SAM (16). However, the in vivo data to support this conclusion are indirect (17) and the fact that CFA synthesis is not affected by a 20-fold change in the intracellular SAM content (7) raises the possibility that other methylene donors may act in vivo. We have tested the possibility of other in vivo donors using the SAMase of phage T3 (10, 12, 24) and report the results of these experiments.

MATERIALS AND METHODS

Bacterial and phage strains and media. Strains FT1 and FT17 are *E. coli* K-12 strains that were described previously (25). Strain FT17 is deficient in CFA synthetase and in CFA synthesis, and strain FT1 is the parent from which it was selected (25). These strains were grown in medium E (27) supplemented with glycerol (0.5%), casein hydrolysate (0.5%), adenine, L-tryptophan, uracil (each at 20 μg/ml) and thiamine (1 μg/ml).

Strain B834 is a suppressor-negative methionine auxotroph of *E. coli* B (24). This strain was provided by F. W. Studier and required an unknown amino acid, which we identified as cysteine. Strain B834 was grown on M9 medium (19) supplemented with glucose (0.4%), L-cysteine (100 μg/ml), L-methionine (20 μg/ml), and thiamine (3 μg/ml). F. W. Studier also provided wild-

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type phage T3 and the SAMase⁻ mutants HR2, a nonsense mutation in gene 0.3, and R1, a deletion of gene 0.3 (24). Lysates of strain B834 infected with each of the phages were assayed for the SAM cleaving enzyme (SAMase) as described by Studier and Movva (24). Only the wild-type phage produced detectable SAMase activity.

Strain K1060 is an *fadE fabB* unsaturated fatty acid auxotroph of *E. coli* K-12 (21). This strain was grown on palmitelaidic acid as previously described for elaidic acid (14). All experiments were done at 37°C with vigorous aeration.

Phospholipid extraction and analysis. The methods used for phospholipid extraction, liposome preparation, differential scanning calorimetry, and fatty acid analysis have all been described previously (14, 15).

Assay of CFA synthesis. The incorporation of L-[*methyl*-^{3}H]methionine into CFA was assayed as previously described (4, 25) except that the L-[*methyl*-^{3}H]methionine was freed of a hydrophobic contaminant (by extraction with chloroform) before use.

RESULTS

Phospholipids were extracted from stationary-phase cultures of the CFA-deficient strain FT17 and its parent strain FT1. The phospholipids of strain FT17 lack CFA (<0.3%), whereas in the phospholipids of strain FT1 about 38% of the acyl chains are CFA (Table 1). Since free CFA have some physical properties that resemble those of their *trans* rather than *cis* unsaturated homologs (2, 9), it has been proposed that the transition of CFA-containing phospholipids should resemble that of phospholipids which contain *trans* unsaturates (13, 18). We, therefore, also prepared *E. coli* phospholipids containing a *trans* unsaturate by growth of strain K1060 on palmitelaidic acid as the required supplement (Table 1). The phospholipids were extracted, dispersed as liposomes, and examined by high resolution scanning calorimetry.

The phospholipids extracted from strains FT1 and FT17 have very similar broad phase transitions that begin at about 5 to 7°C and end at 24 to 25°C (Fig. 1). These transitions are very different from that of the palmitelaidate-containing phospholipids, which give a sharp transition extending from 23 to 35°C (Fig. 1). The order to disorder transition of CFA-containing phospholipids is therefore much more similar to that of phospholipids containing *cis* rather than *trans* unsaturated fatty acids. We, therefore, conclude that the conversion of unsaturated acyl moieties to their cyclopropane derivatives does not significantly alter the order to disorder transition behavior of the membrane phospholipids. It should be noted that the order to disorder transitions detected calorimetrically in phospholipid dispersions are very similar to those observed in intact cells and in isolated membranes (15).

Is SAM the methylene donor in CFA synthesis? The only in vivo evidence that SAM donates the methylene group that forms the cyclopropane ring are the data of Law and coworkers (17). These workers showed that the cyclopropane ring could be labeled with methyl-labeled methionine and that addition of propionate, formate, serine, or glycine to the growth medium did not decrease the extent of labeling with methionine (17). We have done a more direct experiment using phage T3.

Phage T3 codes for an enzyme (SAMase) that cleaves SAM into 5′-methylthioadenosine and homocysteine (10, 12, 24). This enzyme, the first protein synthesized after T3 infection (24), is thought to play a role in host restriction-modification of the phage DNA and is coded by the phage T3 gene 0.3 (24). Nonsense and deletion mutants in gene 0.3 fail to form detectable SA-

TABLE 1. *Percentage (wt/wt) of fatty acid composition of the phospholipids of E. coli FT1, FT17, K1060*

Fatty acid ester	FT1[a]	FT17[a]	K1060
Myristic	3.1	6.6	6
Palmitic	39.0	37.9	16
Palmitoleic	9.2	39.0	76[b]
Methylene hexadecanoic	32.6	<0.3	<0.1
cis-Vaccenic	10.8	16.2	<0.1
Lactobacillic	5.3	<0.1	<0.1

[a] Strains FT1 and FT17 were grown at 37°C to a density of 1.4 × 10^9 cells per ml and harvested after 2 h in stationary phase.

[b] The 16-carbon unsaturate in K1060 was actually the *trans* isomer, palmitelaidic acid.

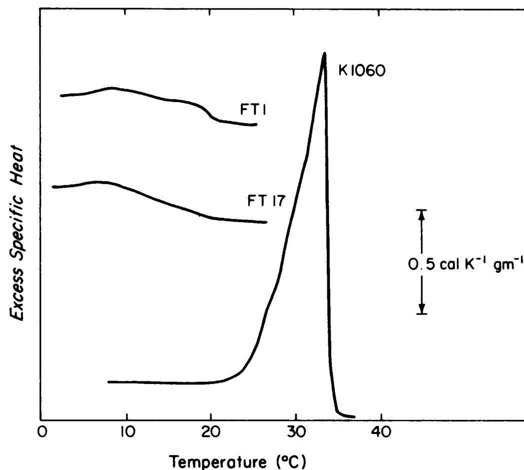

FIG. 1. *Differential scanning calorimetry of phospholipids of strains FT1 and FT17 and of K1060 grown with palmitelaidic acid. The excess specific heat versus temperature is plotted.*

Mase (12, 24). We have, therefore, tested the effect of phage T3 on CFA synthesis. If SAM is the direct donor for CFA synthesis, infection with phage T3 should result in a rapid decrease in the rate of CFA synthesis. The SAMase of phage T3 acts only on SAM, not on methionine or a variety of other compounds (10).

Infection of a methionine auxotroph of *E. coli* with wild-type phage T3 resulted in a rapid decrease in the rate of incorporation of [*methyl*-^{3}H]methionine into CFA. The rate of CFA synthesis was inhibited over 80% during a 4-min labeling period begun 1 min after infection. The rate of [*methyl*-^{3}H]methionine incorporation into CFA continued to decrease until the rate just before lysis was <2% of the normal (uninfected) rate (Fig. 2). Mutants of phage T3 which lack detectable SAMase activity (due to nonsense or deletion mutations of gene 0.3) apparently cause a slight inhibition of CFA synthesis (Fig. 2). This apparent inhibition is not due to residual SAMase activity in these mutants (24; Materials and Methods), but rather can be attributed to an effect of phage T3 on methionine accumulation since methionine incorporation into protein is also inhibited. The rate of methionine incorporation into protein in cells infected with either wild-type or SAMase^{-} phage is inhibited about 40% relative to uninfected cells early after infection (Table 2). Phage T3 and the closely related (albeit SAMase^{-}) phage T7 are known to alter the permeability and transport properties of the host cell early in infection (1, 3, 22), and thus an inhibition of methionine transport was to be expected. The rapid and virtually complete inhibition of CFA synthesis in cultures infected with T3 wild type can therefore be attributed to the induction of the phage-coded SAMase.

DISCUSSION

It has been proposed that the conversion of the *cis* unsaturated acyl moieties of phospholipids to their CFA derivatives could alter the order to disorder phase transition of the phospholipids (13, 18). It was postulated that the properties of the CFA-containing phospholipids should resemble those of phospholipids which contain *trans* unsaturated fatty acids (13). Our data demonstate that this proposal is incorrect. The phase transition of phospholipids which contain CFA is very similar to that of phospholipids containing the homologous unsaturated fatty acids (Fig. 1). Furthermore, the behavior of the CFA-containing phospholipids is very different from that of phospholipids which contain a *trans* unsaturated fatty acid (Fig. 1). A direct comparison of the scans is not completely valid due to the greater homogeneity of the palmitelaidate-

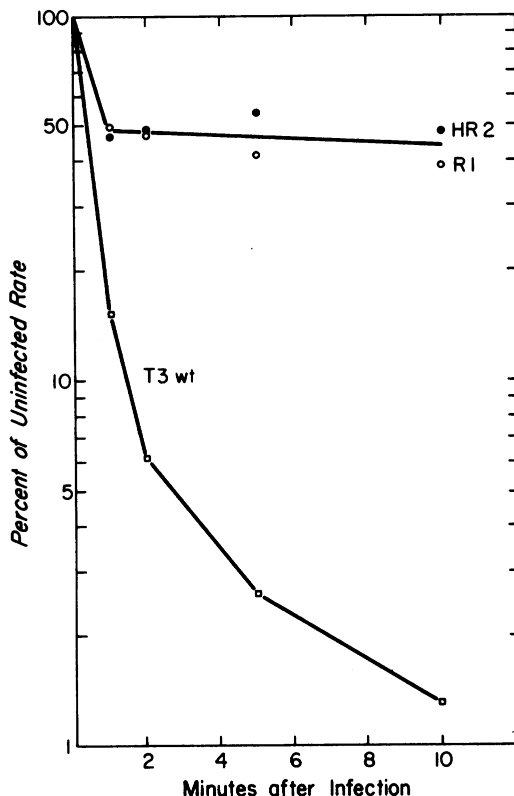

FIG. 2. *CFA synthesis after infection with various T3 phages. Cultures of strain B834 were grown to 5 × 10*^{8} *cells per ml as described in the text. At the times given, before (zero time) or after infection (multiplicity of infection = 10), 0.8-ml samples were incubated with* L-*[methyl-*^{3}*H]methionine (50 µCi/µmol) in parallel with the original culture. After 4 min of incubation, 3 ml of methanol-chloroform (2:1, vol/vol) was added, and the phospholipids were extracted. The phospholipid extract was washed to remove nonlipid contaminants, and the amount of CFA synthesis was quantitated by scintillation counting. The data shown are the mean of two experiments which gave very similar results. The zero time (uninfected) values were 14,000 to 16,000 cpm/sample. Upon lysis of the cultures infected with the mutant phages, the rate of CFA synthesis declined to <2% of the normal rate.*

TABLE 2. *Incorporation of [methyl-*^{3}*H]methionine into protein*[a]

Phage infection	Incorporation (cpm/ml)
None	8,610
T3 wild type	5,490
T3 mutant R1	4,850

[a] Strain B834 (either infected or uninfected) was incubated with [*methyl*-^{3}H]methionine for 4 min after 3 min of infection as described in the legend to Fig. 2. Incorporation into protein was determined by a filter disk assay (19).

enriched lipids. However, the scans of the CFA-containing phospholipids are very similar to those of the *cis* unsaturated fatty acid-containing phospholipids and show none of the characteristics of the *trans* unsaturated fatty acid-containing phospholipids (Fig. 1). Our results agree with other physical studies (21, 26) which showed that phospholipids which contain *cis* CFA pack in a manner indistinguishable from that of phospholipids containing the homologous *cis* (but not *trans*; 21) unsaturated fatty acids. The physical studies, therefore, provide no means to rationalize the synthesis of CFA by bacteria.

Our results using phage T3 and its SAMase negative mutants are the most direct in vivo evidence that SAM is the direct methyl donor for CFA synthesis. The best previous evidence that SAM is the methyl donor was in vitro enzymatic data (16). The in vivo data consisted of the competition experiments of Law and co-workers (17) discussed above and the studies by O'Leary (20) on certain methionine auxotrophs of *Enterobacter aerogenes*. The growth of these auxotrophs could be supported by an exogenous supply of SAM, and addition of exogenous methyl-labeled SAM resulted in the production of radioactive CFA (22). However, 5′-methylthioadenosine or a mixture of adenine and *S*-ribosylmethionine also support the growth of these auxotrophs (23), and thus the studies of O'Leary (20) fell short of demonstrating that SAM is the in vivo methyl donor for CFA synthesis. Our results with the phage T3 SAMase, when coupled with the previous in vitro results of Law and co-workers (16) and with our finding (25) that mutants deficient in CFA synthesis are also deficient in an enzyme (CFA synthetase) which utilizes SAM as the in vitro donor, leave little doubt that the methyl group of SAM is the direct carbon donor for CFA synthesis in vivo.

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