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Cyclopropane Fatty Acid Synthase of *Escherichia coli*. Stabilization, Purification, and Interaction with Phospholipid Vesicles†

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ABSTRACT: The cyclopropane fatty acid (CFA) synthase of *Escherichia coli* catalyzes the methylenation of the unsaturated moieties of phospholipids in a phospholipid bilayer. The methylene donor is *S*-adenosyl-L-methionine. The enzyme is loosely associated with the inner membrane of the bacterium and binds to and is stabilized by phospholipid vesicles. The enzyme has been purified over 500-fold by flotation with phospholipid vesicles and appears to be a monomeric protein having a molecular weight of about 90 000. The enzyme binds only to vesicles of phospholipids which contain either unsaturated or cyclopropane fatty acid moieties. CFA synthase is active on phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin, the major phospholipids of *E. coli*, and also has some activity on phosphatidylcholine. The enzyme is equally active on phospholipid vesicles in the ordered or the disordered states of the lipid phase transition. Studies with a reagent that reacts only with the phosphatidylethanolamine molecules of the outer leaflet of a phospholipid bilayer indicate that CFA synthase reacts with phosphatidylethanolamine molecules of both the outer and the inner leaflets of phospholipid vesicles.

Cyclopropane fatty acids (CFAs)[1] are found in the phospholipids of many eubacteria (Goldfine, 1972) and have also been reported in a few eucaryotic organisms (Christie, 1970).

In bacteria, Law and his co-workers (Law et al., 1963; Zalkin et al., 1963; Chung & Law, 1964a,b) have shown that CFAs are formed by methylenation of the double bond of unsaturated fatty acids [for a review see Law (1971)]. These workers also demonstrated an enzyme, CFA synthase, in *Clostridium butyricum* that catalyzed methylenation of the unsaturated fatty acid moieties of phospholipids using the methyl carbon of *S*-adenosyl-L-methionine (SAM) as the methylene donor. Only phospholipid-bound unsaturated fatty acyl groups were substrates for this enzyme. Free fatty acids, CoA esters, and glycerides were not substrates (Thomas & Law, 1966). The mechanism proposed by Law and co-workers is well supported by data obtained in vivo (Cronan et al., 1974, 1979).

CFA synthase is one of the few enzymes known to act on the nonpolar portion of phospholipids dispersed in a vesicle. The substrate of the enzyme is the double bond of a phospholipid unsaturated fatty acid residue (Law, 1971). This double bond must be 9–11 carbon atoms removed from the glycerol backbone of the phospholipid molecule (Marinari et al., 1974; Ohlrogge et al., 1976), and, therefore, the site of action is well within the hydrophobic region of the lipid bilayer. For these reasons, CFA synthase seems an unusually interesting system for the study of protein–lipid interactions. However, the study of this enzyme has been hampered by its extreme lability. Chung & Law (1964a) were able to purify the *C. butyricum* CFA synthetase only about 50-fold with a low yield. Further purification was not obtained despite extensive efforts (J. H. Law, personal communication).

In this paper we report that the CFA synthase of *Escherichia coli* is greatly stabilized by phospholipid vesicles and can be purified over 500-fold by virtue of its interaction with vesicles. We also report experiments on the intracellular location of the enzyme and on the topology and specificity of its interaction with phospholipid vesicles.

Materials and Methods

Bacterial Strains and Media. *Agrobacterium tumefaciens* ATCC 4452 was obtained from the American Type Culture Collection. *E. coli* K12 strains FT1 and FT17 were described previously (Taylor & Cronan, 1976). Frozen pastes of *E. coli* B (3/4 or full log) were purchased from Grain Processing Co. *A. tumefaciens*, *Bacillus subtilus* strain 168, and the *E. coli* strains were grown on the minimal medium described previously (Taylor & Cronan, 1976) supplemented with 10 g/L each of casein hydrolysate and yeast extract.

Preparation of Phospholipid Vesicles. The various bacteria were grown to maximum turbidity in batches of 15 L (in a 20-L carboy) under vigorous aeration at 10 (*E. coli*) or 30 °C (*A. tumefaciens*). The cells were collected by centrifugation and extracted by the Ames (1968) modification of the method of Bligh & Dyer (1959). The resulting chloroform phase was dried under vacuum on a rotary evaporator and dissolved in a minimal volume of benzene, and the phospholipids were purified by a modification of the method of Law & Essen (1969). The benzene solution was filtered through a scintered glass funnel and evaporated under nitrogen. This residue was dissolved in a minimal volume of chloroform, and 10 volumes of acetone was added. The resulting phospholipid precipitate (freed of neutral lipids) was collected by centrifugation, dissolved in chloroform, flushed with nitrogen, and stored at 4 °C.

Hydrogenations of the phospholipids (50–100 mg) were performed with 5 mg of Adams catalyst (PtO_2) in 20 mL of tetrahydrofuran–methanol (1:1) in an apparatus made from two 125-mL side-arm Erlenmeyer filter flasks connected via the side arm with thick-wall rubber tubing. One flask con-

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[1] Abbreviations used: CFA, cyclopropane fatty acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

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tained the phospholipid solution and the catalyst, and the second flask contained 50 mL of 5 N HCl. The first flask was stoppered with a silicone stopper pierced by a glass tube, to the external end of which was wired a rubber policeman. The second flask was capped with a rubber septum. A stabilized $NaBH_4$ solution (Feiser & Feiser, 1967) was injected into the acid of the second flask which resulted in the immediate evolution of H_2 (monitored by expansion of the rubber policeman). The apparatus was shaken at room temperature until no further uptake of H_2 occurred. Hydrogenation under these conditions has no effect on cyclopropane rings (Christie, 1970).

For convenience we will abbreviate the CFA deficient phospholipids from strain FT17 as CFA^-. If the unsaturated acyl groups have been converted to saturated acids by hydrogenation, the phospholipids are termed $CFA^-(H_2)$. Phospholipids from stationary phase cells of the wild-type strain FT1 are called CFA^+ whereas hydrogenated FT1 lipids are termed $CFA^+(H_2)$. The fatty acid compositions of these lipids are given (Table III).

Three different types of phospholipid vesicles were prepared from these lipids. All were stored at 4 °C under nitrogen and the CFA^- vesicles were active as substrates for at least 1 month when stored under these conditions. Storage at room temperature resulted in a rapid loss of activity. The concentration of phospholipid in the vesicle preparations was determined by the hydroxamate test (Shapiro, 1953).

Sonicated vesicles were prepared as follows. A solution of phospholipd in chloroform was evaporated to dryness under a stream of nitrogen in a metal sonication vessel. Distilled water (1 mL/10 mg of lipid) was added to the vessel, and the lipid was dispersed by sonic oscillation for 1 min using a Branson Model W140 sonifier (1/2-in. horn). During resuspension the temperature of the solution was allowed to rise from 20 to 50–60 °C. Single bilayer liposomes were prepared essentially as described by Deamer & Bangham (1976). Multilamellar liposomes were prepared as described for sonicated vesicles except that vigorous agitation and homogenization were used rather than sonication to disperse the lipid.

CFA Synthase Assay. The lipid substrates used for the assay were sonicated dispersions or multilamellar liposomes made from CFA^- phospholipids. The assay mixture (0.1-mL final volume) contained 0.1 mg of a CFA^- phospholipid dispersion; potassium phosphate (pH 7.5), 2 μmol; [*methyl*-^{3}H]-*S*-adenosyl-L-methionine (SAM), 0.05 μmol, at a final specific activity of 25 μCi/μmol; and 0.1 unit of *S*-adenosyl-L-homocysteine hydrolase (SAHase) (see below). The carrier SAM was Sigma type I and the tritium-labeled SAM was from New England Nuclear. The reaction was quantitated by measuring the incorporation of label from SAM, which is soluble in hot trichloracetic acid (Cl_3AcOH), into Cl_3AcOH insoluble lipid using a modification of the filter disk assay of Goldfine (1966). After incubation at 37 °C for 30 min, the entire reaction mixture was pipetted onto a 2.4-cm (in diameter) disk of Whatman 3 MM filter paper mounted on a pin. The filter disks were dried in a stream of hot air for 20 s and immersed in Cl_3AcOH (10% w/v) for 5 min at room temperature. The disks were then placed in a boiling solution of 5% (w/v) Cl_3AcOH for 5 min. Following treatment with the boiling Cl_3AcOH solution, the disks were washed in two changes of distilled water for 15 min each and then dried and assayed for radioactivity in Aquasol I scintillation fluid (New England Nuclear Corp.). The assay is linear with protein from 0.02 to 5 mg of crude supernatant protein and is linear with time for at least 1 h. A unit of CFA synthase activity is defined as 1 pmol of CFA formed per min at 37 °C. In many cases the protein concentration of purified enzyme fractions could not be determined due to a combination of the low concentrations of protein involved and the presence of interfering substances (lipid vesicles, reducing agents). However, in such cases the assays were always done at several dilutions of enzyme to establish the linearity of the assay. It is important that the [^{3}H]SAM and carrier SAM be mixed just before use. Storage of concentrated [^{3}H]SAM solutions results in formation of a Cl_3AcOH insoluble product which leads to high background values.

Partial Purification of SAH Nucleotidase (SAHase). This enzyme was purified from *E. coli* B by essentially the method of Duerre (1962). SAHase preparations purified by ammonium sulfate fractionation and DEAE-cellulose chromatography were free of CFA synthase activity. It was found that less-purified preparations could be freed of CFA synthase with full retention of SAHase activity by heating to 45 °C for 15 min. SAHase preparations were stored at −20 °C in 50 mM Tris-HCl (pH 7.5) buffer and were stable for at least 1 year. SAHase activity was assayed as described by Duerre (1962) except Nelson's (1944) test was used to measure the production of reducing sugar. One unit of SAHase activity is defined as 1 μmol of reducing sugar formed from SAH per min at 37 °C and pH 7.5. SAHase activity could also be assayed indirectly by relief of the inhibition of CFA synthase by added SAH. SAH was purchased from Sigma.

Partial Purification of CFA Synthase. Two procedures were used to partially purify CFA synthase. The early steps of both purification schemes were identical. The starting material was frozen cell pastes of *E. coli* B (full or 3/4 log) purchased from Grain Processing Co. With one exception (which had no activity), various lots of these cells were found to have the same activity as freshly grown cells of *E. coli* K12 or B. All steps were done at 0–4 °C.

The *E. coli* B paste (20 g) was thawed and homogenized in 20 mL of 50 mM sodium phosphate buffer, pH 7.6, which contained $MgCl_2$ (5 mM) and about 1 mg of deoxyribonuclease I. The cells were disrupted by two passages through a French pressure cell at 11 000 psi. The resulting lysate was cleared of large particulate material by centrifugation at 10000*g* for 10 min, and the supernatant was retained. The centrifugation supernatant was diluted to a protein concentration of 10 mg/mL, and ammonium sulfate (Mann Enzyme Grade) was slowly added to 40% of saturation. After equilibration the precipitate was collected by centrifugation at 10000*g* for 15 min and dissolved in the phosphate buffer. Residual ammonium sulfate was removed either by dialysis or by gel filtration on Sephadex G-25.

Method A. In method A, substantial purification of the enzyme could be achieved by sedimentation with phospholipid vesicles. CFA synthase purified through the ammonium sulfate step is mixed with sonicated CFA^- phospholipid vesicles and centrifuged at 100000*g* for 1 h. The final concentrations of protein and lipid were 20 and 1 mg/mL, respectively. The pellet resulting from the centrifugation was then dispersed in phosphate buffer, and KCl was added to a final concentration of 1 M while cooling the solution in a salt–ice water bath. This solution was centrifuged at 100000*g* for 1 h and the supernatant, which contained the CFA synthase, was dialyzed to remove the KCl. This preparation was over 60-fold purified and was dependent on exogenous phospholipid for activity.

Method B. Due to erratic results with method A, method B was developed to exploit the affinity of CFA synthase toward

phospholipid vesicles. In method B, the low density of multilamellar vesicles was exploited to flotate CFA synthase. Ammonium sulfate purified enzyme, 60% sucrose, and a suspension of multilamellar liposomes of CFA^- lipids were mixed to give a solution containing final concentrations of sucrose, protein, and liposomes of 30% (w/v), 10 mg/mL, and 4 mg/mL, respectively, in 50 mM sodium phosphate (pH 7.5). After incubation at 37 °C for 15 min, 4 mL of this mixture was placed in a centrifuge tube and sequentially overlaid with 0.5 mL of phosphate buffer containing 25% (w/v) sucrose (d = 1.09 g/mL), 0.5 mL of phosphate buffer containing 20% (w/v) sucrose (d = 1.08 g/mL), and 0.1 mL of buffer. The tube was then centrifuged at 80000g for 2 h. After centrifugation, the lipid was visible as an opalescent band in the 20% sucrose layer. This layer was removed by puncturing the side of the tube and removing the band with a syringe.

In our later experiments we refined this purification by first doing a negative purification using $CFA^-(H_2)$ liposomes, to which CFA synthase does not bind. The flotation was done as above except that $CFA^-(H_2)$ liposome lipids were used. After centrifugation the 30% sucrose layer was removed through the bottom of the tube, mixed with CFA^- liposomes, overlaid with layers of 25 and 20% sucrose, and centrifuged as above.

To remove the lipids from the CFA synthase, the vesicle-bound synthetase was mixed with sufficient sucrose and KCl to give concentrations of 40% (w/v) and 1 M, respectively. This mixture was centrifuged at 50000g for 1 h. Centrifugation resulted in the lipid being layered on the surface of the solution, and lipid-free CFA synthase was obtained by puncturing the bottom of the tube.

After removal of KCl by dialysis or gel filtration, the enzyme fraction obtained by either method was stored at −70 °C in the presence of 1 mg/mL sorbitan monolaurate liposomes. This compound greatly stabilizes the enzyme but can be readily separated from the enzyme by centrifugation at 50000g for 15 min.

Lipid Analyses. Fatty acid esters were quantitated by the hydroxamate method (Shapiro, 1953) using lauric hydroxamate as standard. Lipid phosphate was determined as described by Ames (1966). Conversion of phospholipid acyl moieties to fatty acid methyl esters and gas chromatography of the methyl esters were done as previously described (Taylor & Cronan, 1976; Jackson & Cronan, 1978). Thin-layer chromatographic separation of phospholipids used the plates and solvents described previously (Tunaitis & Cronan, 1973). For the separation of TNBS–PE from PE and PG, the solvent system was chloroform–methanol–3.5 N ammonia (65:40:40 v/v).

Protein Estimation. Protein concentrations were determined by either the Lowry (Lowry et al., 1951) or the microbiuret (Munkres & Richards, 1966) procedures using crystalline bovine serum albumin as the standard.

Results and Discussion

Intracellular Localization of CFA Synthase. Cox et al. (1973) reported the presence of CFA synthase in membrane vesicles prepared from *E. coli*. However, no data were given as to what fraction of the total activity was associated with the membrane nor in which of the cellular membranes (inner or outer) the enzyme was located. We analyzed the distribution of CFA synthase in subcellular fractions obtained by the methods of Heppel et al. (1962) and of Schnaitman (1970). We found no CFA synthase activity in either the outer membrane or the periplasmic space; all of the activity was distributed between the inner membrane and the soluble fractions. The portion of enzyme associated with the inner membrane depended on the protein concentration of the lysate before centrifugation, indicating a loose association with the inner membrane. A loose association was also indicated by the quantitative elution of the membrane-associated activity from the membrane with 1 M NaCl.

Table I: CFA Synthase Levels in Cells of Various Phases of Growth[a]

growth phase	CFA/(CFA + UFA)	CFA synthase (units/mg of protein)
early log (0.2)	0.24	33.3
early stationary (1.3)	0.49	33.5
stationary (4.3)	0.50	44.9
late stationary (7.0)	0.66	15.8

[a] Strain FT1 was grown on glucose minimal medium (Taylor & Cronan, 1976) supplemented with casein hydrolysate (1%) at 37 °C from an inoculum of 5×10^5 cells/mL. At the phases of growth given, samples were removed and the cells harvested by centrifugation. A portion of each sample was taken for lipid extraction, and the remainder was disrupted in a French pressure cell, freed of intact cells by a low-speed (50000g) centrifugation, and assayed for CFA synthase activity. The numbers in parentheses are the cells per 10^{-9} mL (determined by turbidity) at the growth phase given. The cyclopropane fatty acid [CFA/(CFA + UFA)] content of the cells is also given.

Stabilization and Partial Purification of CFA Synthase. Cronan (1968) and reported that CFA synthase levels were similar in early log phase cultures and stationary phase cultures. This was surprising because CFAs are preferentially synthesized as cultures enter the stationary phase (Law et al., 1963; Cronan, 1968). We have repeated Cronan's (1968) experiment using our more sophisticated assay and have obtained very similar results (Table I). We also found that commercially grown *E. coli* cells had activities similar to those obtained from cells grown in the laboratory and thus have used commercially grown cells as our major enzyme source.

Our first attempts to purify CFA synthase were plagued with large losses of activity in the course of conventional purification procedures such as ion-exchange and gel filtration chromatography. The behavior of the *E. coli* enzyme was therefore reminiscent of that of the *C. butyricum* CFA synthase. During a study of the stability of the *E. coli* CFA synthase, we found that the activity in crude extracts was quite stable whereas the activity in the supernatant of such extracts which had been centrifuged at 100000g for 2 h was very labile (Table II). All activity in these supernatants was lost during a 30-min incubation at 37 °C, and half the activity was lost during overnight storage at 4 °C (Table II). However, enzyme assays performed at 37 °C using the supernatant enzyme were linear with time for over 1 h. It, therefore, seemed that a component(s) of the assay stabilized CFA synthase. Phospholipid vesicles seemed the most likely assay component to confer stability because such vesicles would be formed during cell breakage (and thus be present in crude extracts) but would be removed by ultracentrifugation. It was also noted that the assay of CFA synthase using crude extracts did not require an exogenous source of lipid whereas the assay using the high-speed centrifugation supernatant had an absolute requirement for added phospholipid vesicles.

For these reasons we tested phospholipid vesicles for their ability to stabilize CFA synthase (Table II). We found that phospholipid vesicles greatly stabilized the enzyme. However, it should be noted that the addition of phospholipid vesicles did not convert CFA synthase to an unusually stable enzyme;

Table II: Stabilization of CFA Synthase[a]

	phospholipid (mg/mL)	other	% of initial act.
expt 1 (4 °C; 16 h)	0		51
	0.025		60
	0.5		100
	0	KCl (0.2 M)	12
	4.0	KCl (0.2 M)	14
expt 2 (37 °C; 30 min)	0		<1
	0.025		20
	0.050		35
	0.10		50
	0.50		80
	2.0		100
	0	SML (0.5 mg/mL)	71
	0	SOL (0.5 mg/mL)	48
	0	SAM (0.1 mM)	<1
	0	glycerol (20%) plus phosphate (0.2 M)	40

[a] The enzyme fractions used were lipid-free preparations resulting from a high-speed centrifugation of a crude extract or from purification method A. SML and SOL are, respectively, the lauryl or oleoyl monoesters of sorbitol dispersed as liposomes. Sonicated CFA^- phospholipid vesicles were used. An activity of 100% was 10 units of activity in experiment 1 and 43 units of activity in experiment 2.

all activity was lost during a 15-min incubation at 50 °C. A variety of detergents were also tested as stabilizers. The commonly used detergents (Tritons, Tweens, Brijs, etc.) destroyed enzyme activity. Two detergents, the monooleate and the monolaurate esters of sorbitol, were found to stabilize the enzyme. Unfortunately, these detergents inhibit the enzyme assay and disperse poorly, thus limiting their usefulness. We also tested SAM, the methylene donor of the reaction, and a variety of molecules often used to stabilize enzymes (phosphate, glycerol, ethylene, glycol, etc.). None of these molecules was effective in stabilizing CFA synthase (Table II). However, a mixture of 20% glycerol and 0.2 M potassium phosphate buffer (pH 7.6) was somewhat effective in stabilizing partially purified (but not highly purified) enzyme preparations (Table II).

The finding that CFA synthase requires the presence of lipid for maintenance of activity explained our activity losses during chromatography since such procedures tend to resolve protein and lipid. The high salt concentrations used in ion-exchange chromatography also destabilized the enzyme activity by dissociating bound phospholipid from the protein. Due to these considerations we developed methods for the purification of CFA synthase which avoided conventional chromatographic steps.

Our rationale for purification was based on the phospholipid stabilization studies discussed above. The stabilization of CFA synthase by phospholipid vesicles and the fact that the vesicles are a substrate for the enzyme suggested that CFA synthase bound rather tightly to vesicles. We therefore determined the effect of the addition of multilamellar vesicles of substrate phospholipids (4 mg/mL) on the sedimentation behavior of the CFA synthase activity. In the presence of multilamellar vesicles (or of smaller sonicated vesicles) of CFA^- lipids, about half of the CFA synthase was sedimented. Addition of SAM somewhat increased the fraction of the activity sedimented (to 70–90%), and high salt concentrations hindered the association. The presence of 1 M KCl decreased the amount of activity sedimented in the presence of lipid vesicles by over 90%. By use of the binding of CFA synthase to lipid vesicles, we were able to purify the enzyme appreciably (method A, Materials and Methods). However, the results were erratic and hence we developed a more refined separation method also based on the binding of CFA synthase to phospholipid vesicles.

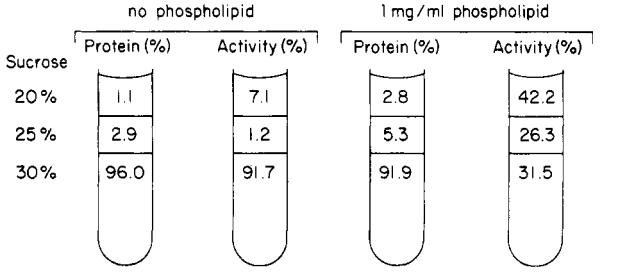

FIGURE 1: Distribution of CFA synthase activity and protein in sucrose step gradients in the absence (left) or presence (right) of 1 mg/mL liposomes formed from CFA phospholipids. The enzyme preparation (10 mg/mL ammonium sulfate purified enzyme) was mixed with liposomes in 30% sucrose overlaid first with 25% sucrose and then with 20% sucrose as described under Materials and Methods. After centrifugation, the 20 and 25% layers were collected through the side of the tube whereas the 30% layer was collected through the bottom of the tube.

Table III: Fatty Acid Composition of Phospholipid Vesicles[a]

	wt %			
methyl ester	CFA^+	$CFA^+(H_2)$	CFA^-	$CFA^-(H_2)$
myristic	3.5	3.1	2.6	5.6
palmitic	35.1	38.8	40.2	72.3
palmitoleic	8.7	<0.5	37.7	<0.5
C17 CFA	30.5	34.7	<0.5	<0.5
stearic	<0.5	15.2	<0.5	22.2
cis-vaccenic	15.9	<0.5	19.1	<0.5
C19 CFA	6.3	7.2	<0.5	<0.5

[a] The compositions were determined by gas chromatography of fatty acid methyl esters (see Materials and Methods). H_2 means hydrogenated. CFA^+ and CFA^- mean phospholipids from strains FT1 and FT17, respectively.

Liposomes ($d \sim 0.89$ g/mL) are much less dense than protein ($d \sim 1.37$ g/mL), and thus association of CFA synthase with a liposome should decrease the density at which the enzyme activity bands in isopycnic sucrose density gradients. In effect, the liposome should float CFA synthase. For preparative purposes we used step gradients (Figure 1) in which a mixture of crude CFA synthase and CFA^- liposomes was mixed in 30% sucrose (w/v; $d = 1.12$ g/mL) and overlaid with two layers of less dense sucrose. After centrifugation in the presence of added liposomes, over half of the activity was found in the upper two layers whereas in the absence of added liposomes less than 10% of the activity was found in the upper layers (Figure 1). In both the presence and the absence of lipid, over 90% of the protein remained in the lower layer. The partial flotation observed in the absence of added lipid can be attributed to residual cellular lipid present in the crude extract.

The effectiveness of liposomes to float CFA synthase depended on the fatty acid composition of the vesicular phospholipids. The liposomes used in the experiments of Figure 1 were composed of CFA^- phospholipids which contain a high proportion of unsaturated fatty acids and no CFA [Table III, Taylor & Cronan (1976)]. Liposomes of CFA^+ phospholipids, which contained little unsaturate but a large amount of CFA (Table III), were equally effective, and some effectiveness (about 30%) remained when the unsaturates were eliminated by hydrogenation (Table III). The enzyme, therefore, has affinity for both unsaturated and cyclopropane phospholipid fatty acyl moieties. In contrast, the enzyme was not floated (<2% flotation) by liposomes made from phospholipids containing only straight-chain [$CFA(H_2)$] or branched-chain

Table IV: Purification of CFA Synthase[a]

step	sp act. (units/mg of protein)	yield (%)
extract	19.1	(100)
centrifugation	39.2	77
$(NH_2)_2SO_4$ precipitation	56.9	65
single flotation	98.0	26
double flotation	1130.0	9

[a] The purification was as given under Materials and Methods. The activities after the flotation purifications are those obtained before removal of lipid with KCl. Single flotation was with CFA^- lipids as described in Figure 1. Double flotation means that the enzyme was first centrifuged in the presence of $CFA^-(H_2)$ lipids and then with CFA^- lipids.

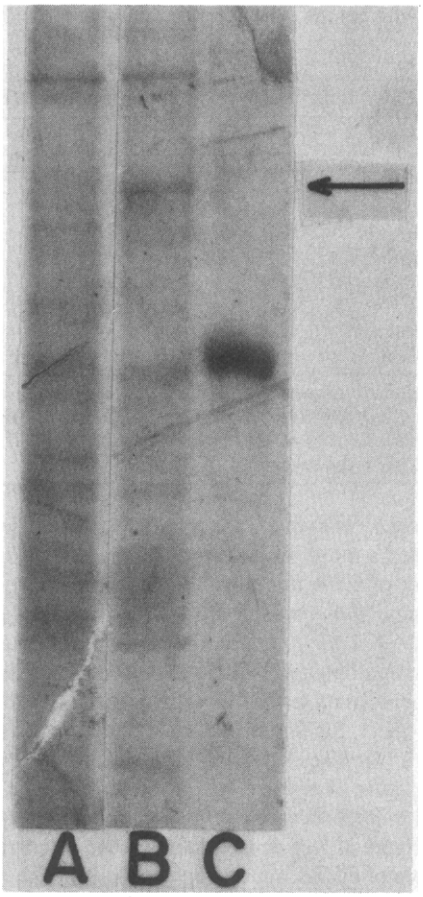

FIGURE 2: Sodium dodecyl sulfate–acrylamide gel electrophoresis of CFA synthase. The gels were 9% acrylamide gels run in the presence of sodium dodecyl sulfate and stained with Comassie blue according to Laemmli (1970). The samples were boiled in the loading buffer before application to the gel. Lane A contains CFA synthase exposed to a single flotation step with $CFA^-(H_2)$ liposomes. Lane B contains CFA synthase purified by double flotation, first with $CFA^-(H_2)$ liposomes and then with CFA^- liposomes. Lane C is bovine serum albumin. See Materials and Methods for further details.

saturated fatty acids (*B. subtilus* phospholipids). In addition, neither of these liposome preparations inhibited the CFA synthase reaction (see below).

These results suggested a plausible scheme for purification of CFA synthase (Table IV). Following $(NH_4)_2SO_4$ fractionation, the enzyme was first mixed with vesicles of $CFA^-(H_2)$ lipids, and the vesicles were removed by flotation. The enzyme that remained in the lowest layer was then mixed with CFA^- phospholipid vesicles, and the flotation procedure was repeated. An appreciable fraction (10–40%) of the enzyme was found in the upper layers of the second flotation,

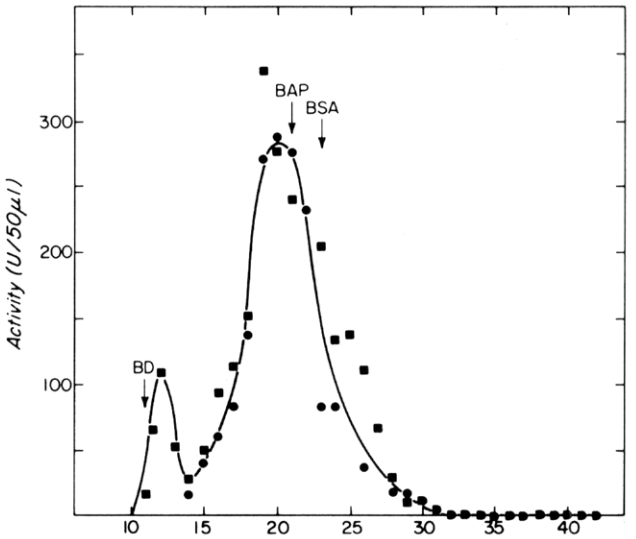

FIGURE 3: Elution profile of CFA synthase from Sephadex G-200. Two runs of the same column are shown. The standards run were Dextran Blue 2000 (BD), *E. coli* alkaline phosphatase (BAP), and bovine serum albumin (BSA). The CFA synthase preparations had been purified either by method A (■) or by centrifugation at 100000*g* (●). The elution buffer was 50 mM Tris-HCl, pH 8, plus 1 mM EDTA. The column volume was 75 mL and 2-mL fractions were collected. The salt peak was at fraction 36.

and this enzyme had been considerably purified (300–600-fold) with respect to the starting material. If the primary flotation was omitted, the purification was considerably less (only 20–80-fold), indicating that the primary flotation removed considerable amounts of non-CFA synthase proteins which had affinity for liposomes (Table IV).

CFA synthase purified over 500-fold with respect to the crude extract was not homogeneous (Figure 2). Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gave a major protein band with an apparent molecular weight similar to that of the σ subunit of the RNA polymerase of *E. coli* (about 90 000). However, the gels also contain several other distinct protein bands as well as a background haze of protein (Figure 2). The purity of these CFA synthase preparations is probably about 10%. The 90 000 molecular weight band is thought to be CFA synthase because (1) the intensity of this band is proportional to the specific activity of the preparation loaded on the gel, (2) 90 000 is the molecular weight of the active enzyme (see below), and (3) very little of this band was seen when flotation was done using liposomes made of saturated (Figure 2) or *B. subtilis* phospholipids whereas it was a major band seen upon a second flotation with liposomes of unsaturated phospholipids (Figure 2).

Molecular Weight of CFA Synthase. The molecular weight of CFA synthase was determined by equilibrium sedimentation in the absence of lipid, followed by enzyme assay. The method used was essentially that of Bothwell et al. (1978). CFA synthase sedimented in a manner very similar to that of *E. coli* alkaline phosphatase and thus has a molecular weight of about 90 000 by assuming a typical partial specific volume (0.725 mL/g) for the protein.

Gel filtration on Sephadex G-200 (Figure 3) also showed a similar migration for CFA synthase and alkaline phosphatase. These results agreed with the sedimentation equilibrium and $NaDodSO_4$ gel results, and when taken together these data suggested that CFA synthase is a globular monomeric protein of about 90 000 daltons.

Catalytic Properties of CFA Synthase. Flotation-purified CFA synthase showed normal saturation kinetics with both substrates, SAM and phospholipid vesicles (Figures 4 and 5).

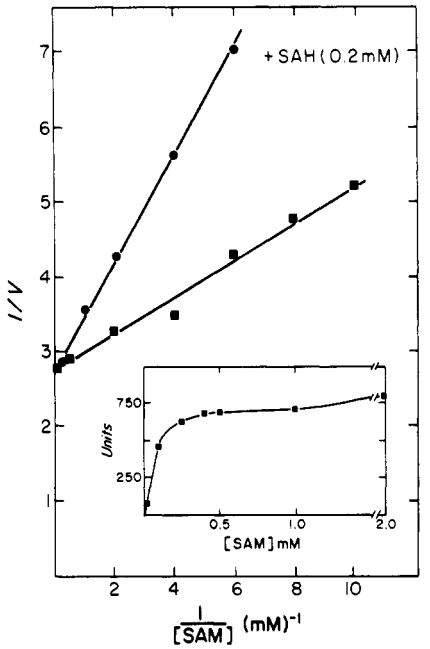

FIGURE 4: Dependence of CFA synthase on SAM concentration. The inset shows the saturation curve of CFA synthase activity with the SAM concentration. Enzyme purified by method B (double flotation) was used. The data from the inset are displayed as a Lineweaver–Burk plot along with data obtained in the presence of 0.2 mM SAH.

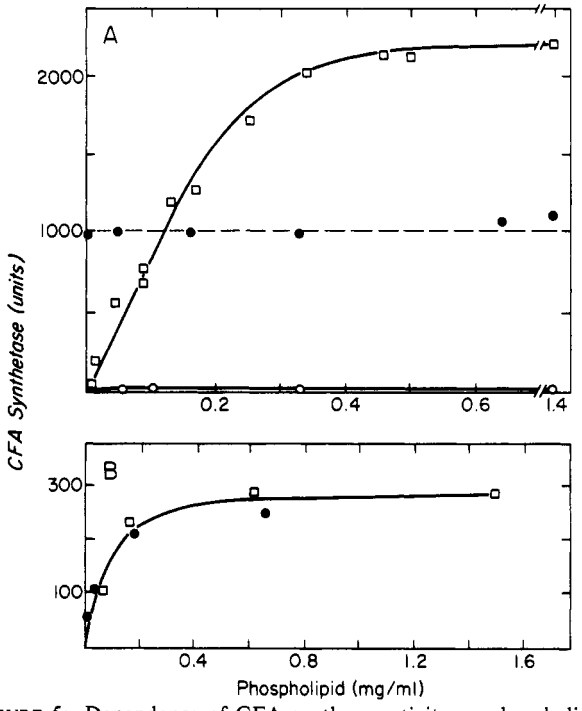

FIGURE 5: Dependence of CFA synthase activity on phospholipid vesicle concentration. In panel A, the lipid vesicles were liposomes made of CFA^- phospholipids (□) or $CFA^-(H_2)$ phospholipids (○). Also shown (●) is the effect of the addition of various concentrations of $CFA^-(H_2)$ liposomes to reaction mixtures containing 0.12 mg/mL CFA^- liposomes. In panel B the lipid vesicles were two different preparations of sonicated CFA^- phospholipids. The enzyme preparation in panel A was a high-speed lipid-free supernatant whereas enzyme purified by method B was used in panel B.

The Michaelis constant for SAM is 90 μM. The reaction was competitively inhibited in respect to SAM by *S*-adenosylhomocysteine (SAH), a product of the reaction (Figure 4). This was expected since Chung & Law (1964b) reported that the CFA synthase of *C. butyricum* was inhibited by SAH and methyltransferases are generally inhibited by SAH (Salvatore et al., 1977). However, the *E. coli* CFA synthase had a K_i of 220 μM and is therefore about 10-fold less sensitive to SAH inhibition than the clostridial enzyme. SAH inhibition of the *E. coli* CFA synthase was completely reversed by addition of *E. coli* SAHase, an enzyme that cleaves SAH to ribosylhomocysteine and adenine (Duerre, 1962), or by addition of adenosine deaminase which converts SAH to *S*-inosylhomocysteine (Zappia et al., 1969). We routinely added SAHase to the assay to preclude inhibition by SAH.

Table V: Reaction of CFA Synthase with Vesicles of Mixed Phospholipid[a]

phospholipid species	nmol of phospholipid present	react. (pmol of CFA formed per nmol of phospholipid)
E. coli phospholipid mixture		
PE	247	28.8
PG	23	43.0
CL	20	37.5
A. tumefaciens phospholipid mixture		
PE	84	38.0
MMPE	73	16.1
DMPE	44	0.5
PC	93	1.7
PG	tr	
CL	tr	

[a] The lipid preparations and assay were as described under Materials and Methods using a lipid-free preparation of enzyme purified by method B. The vesicles were formed by sonication.

CFA synthase was saturated with phospholipid vesicles at a liposome concentration of about 0.67 mM (0.5 mg/mL) of phospholipid (Figure 5A). Phospholipids dispersed by sonication were somewhat more effective on a weight basis (Figure 5B). This is probably due to the greater external surface area per weight of lipid of single bilayer vesicles. However, the heterogeneous size of sonicated vesicles made from *E. coli* lipids (Jones & Osborn, 1977; Fung et al., 1979) and the unusual mechanism of action of CFA synthase (see below) precluded calculation of a Michaelis constant. Liposomes made of $CFA^-(H_2)$ lipids were inactive substrates and failed to inhibit the reaction of the enzyme with CFA^- liposomes (Figure 5B).

CFA synthase is sensitive to sulfhydryl reagents. Dithiobis(nitrobenzoic acid) (Nbs_2), *N*-ethylmaleimide, and *p*-(hydroxymercuri)benzoate all inhibited the enzyme; however, iodoacetic acid did not. Nbs_2, the most specific of these reagents, inhibited the enzyme over 90% at a concentration of 0.5 mM. Inhibition by Nbs_2 was completely reversed by addition of a reducing reagent such as dithiothreitol at 2 mM. In fact, CFA synthase was bound to a column of immobilized Nbs_2 (Lin & Foster, 1975), and a portion of the bound enzyme could be eluted with reducing agents (Taylor, 1977). CFA synthase could be partially protected from Nbs_2 inhibition by high concentrations of SAM (Taylor, 1977).

Substrate Specificity. In CFA^- vesicles containing the mixture of phospholipids found in *E. coli*, CFA synthase reacted with each of the phospholipid species present (Table V). Most of the CFA formed was found in phosphatidylethanolamine (PE), the major phospholipid of *E. coli*. However, on a molar basis the substrate activity of PE was slightly lower than that of the two other *E. coli* phospholipids, phosphatidylglycerol (PG) and cardiolipin (CL). This behavior is consistent with the relative proportions of CFA in the various phospholipids found in stationary phase cells (Cronan, 1968).

Table VI: Individual *E. coli* Phospholipid Species as Substrates for CFA Synthase[a]

phospholipid species	concn of phospholipid in reaction (mg/mL)	act. (units)	rel react. (units/mg of phospholipid)
total *E. coli* phospholipids	2	178.5	89
PE	0.5	39	78
PG	0.2	71	355
CL	0.1	0	0
PE + PG	0.5 + 0.1	118	197

[a] A lipid-free preparation of enzyme prepared by method B was used. The PE preparation was mixed with a small amount of PG to facilitate dispersion. Sonicated vesicles were used.

It should be noted that the fatty acid compositions of the phospholipids of *E. coli* are quite similar (Cronan & Vagelos, 1972).

We have also tested if CFA synthase reacted with various phospholipids that are not found in *E. coli* (Table V). We used vesicles made from the phospholipids of *A. tumefaciens*, a bacterium which contains phosphatidylcholine (PC) and the mono- and dimethyl derivatives of PE in addition to PE. The fatty acid compositions of the various phospholipids of this organism are quite similar to that of PC since they are synthesized by methylation of the amino group of PE (Kaneshiro & Law, 1964). The PE of *A. tumefaciens* was as active a substrate as *E. coli* PE; however, the reactivity of the methyl-PE derivatives was considerably less. Dimethyl-PE was essentially inactive whereas monomethyl-PE and PC were less active than PE.

Preliminary results indicate that the substrate activities of the component phospholipids of *E. coli* differed when each was dispersed alone into a vesicle (Table VI). PG was the most active substrate when dispersed alone, CL was completely inactive, and PE had an activity similar to that found in mixed vesicles. Addition of sodium dodecyl sulfate or $CaCl_2$ did not increase the activity of these vesicles. We have also tested a variety of other lipids. Egg yolk PC had an activity similar to that of *A. tumefaciens* PC whereas phosphatidylserine (bovine) and phosphatidylinositol (plant) were inactive. A detergent, sorbitan monooleate, that stabilized CFA synthase (Table II) was also not a substrate. At a lipid concentration identical with that of the substrate, PC (dicaproyl) and phosphatidylserine (dipalmitoyl) inhibited CFA synthase over 95% (data not shown), suggesting that some inactive phospholipids bind to the enzyme.

CFA synthase was active on the residual unsaturates found in *E. coli* phospholipids in which CFA synthesis had proceeded to the maximal extent in vivo (Table III). The lack of conversion of the residual UFA molecules found in vivo is therefore not a property of the phospholipid molecule per se. In vitro as well as in vivo, CFA synthase converts both palmitoleoyl and *cis*-vaccenoyl residues to their CFA derivatives (Cronan, 1968; Taylor, 1977).

Effect of the Lipid Phase Transition on Substrate Activity. The activities of several enzymes which interact with lipid are known to be affected by the physical properties of the lipid moiety (Gennis & Jonas, 1977). Phospholipids in a lipid bilayer undergo a thermotrophic phase transition in which the acyl chains of the phospholipids go from a rather rigid, ordered structure at low temperature to a fluid, disordered structure at elevated temperatures. Since CFA synthase converts the double bond of a phospholipid unsaturate moiety to a cyclopropane ring, this enzyme interacts with that portion of a

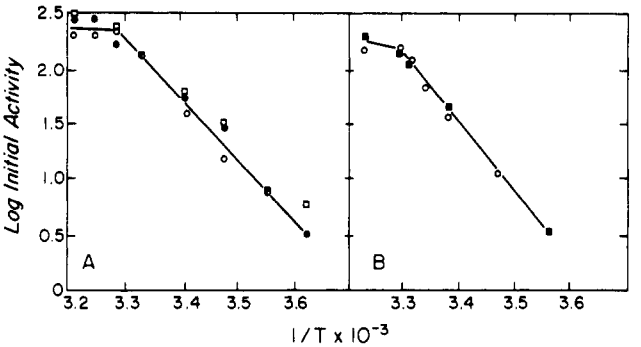

FIGURE 6: The effect of temperature on the CFA synthase reaction on vesicles of different fluidity. The vesicles used were multilayer liposomes prepared from the phospholipids of strains altered in UFA synthesis (Cronan & Gelmann, 1973). The lipid phase transitions were determined on these same lipid preparations by Jackson & Cronan (1978). The ranges of the transitions as reported in that paper were (●) 0–6, (□) 10 to 15–47, and (■) 3–37 °C. Two different experiments using these liposomes and CFA synthase (lipid free) purified by method B are shown. The assay temperatures were (left to right) 42, 37, 32, 27, 22, 15, 8, and 0 °C.

phospholipid bilayer that undergoes the greatest alteration during the lipid phase transition (Seelig & Seelig, 1977).

We have therefore examined the rate of the reaction of CFA synthase with vesicles which contained various proportions of ordered and fluid lipid. The relative amounts of ordered and fluid lipid were varied both by temperature and by altering the ratios of saturated to unsaturated fatty acids in the vesicular phospholipids. An increase in the amount of unsaturated fatty acid decreases the temperature of the range over which lipid phase transition occurs. In our experiments, vesicles were made from phospholipids extracted from *E. coli* cultures in which the amount of unsaturated fatty acid had been manipulated by genetic means (Cronan & Gelman, 1973; Jackson & Cronan, 1978). The thermotrophic lipid phase transitions of these vesicles were then determined by differential scanning calorimetry using the Privalov calorimeter, a high-resolution instrument (Jackson & Cronan, 1978). The lipid phase transitions of the vesicles varied widely. For example, the temperature at the high-temperature end of the transition (all chains fluid) varied from 6 to 47 °C. The range of the transitions are given in the legend to Figure 6; the actual calorimetric scans and fatty acid composition have been published elsewhere (Jackson & Cronan, 1978). The initial rate of the reaction of CFA synthase with each of these vesicle preparations was examined at a number of temperatures ranging from 0 to 42 °C. As shown in Figure 6, CFA synthase was not sensitive to the order–disorder phase transition of the substrate lipids. The substrate activities of the different vesicle preparations were very similar. The slopes of the Arrhenius plots for each vesicle preparation were also nearly identical with an activation energy of about 25 kcal/mol and a discontinuity between 32 and 34 °C. These results, therefore, indicate that the CFA synthase reaction is not affected by the order–disorder state of the lipid substrate.

Topology of the CFA Synthase Reaction. As *E. coli* enters the stationary phase of growth, about 80% of the 16 carbon unsaturated fatty acid moieties are converted to their cyclopropane derivatives (Table III). Since palmitoleic acid is a component of most of the phospholipid molecules of the cell, CFA synthase is somehow able to react with the lipids of both monolayer leaflets of the bilayers of both cellular membranes. These considerations, therefore, suggested that in vitro CFA synthase might react with phospholipid molecules on both the inner and the outer leaflets of single bilayer vesicles.

Table VII: Resistance of CFA-Labeled Phospholipids to TNBS[a]

additions	time of incubation (min)	^{3}H (CFA) radioact. (counts/5 min) in PE	TNBS-PE	PG	^{3}H (CFA) label [PE/(PE + TNBS-PE)]	^{14}C (phospholipid) radioact. (counts/5 min) in PE	TNBS-PE	PG	^{14}C (PE) label [PE/(PE + TNBS-PE)]
expt 1									
none	15	865	2163	506	29	5295	5319	3322	50
none	45	2164	5574	853	28	6576	6851	3676	49
none	180	4032	7781	1131	34	5658	5779	3124	49
0.4% Triton X-100	180	595	5621	3479	6	737	4470	2641	13
expt 2									
TNBS at 0 min	40	1402	2859	990	34	5777	7467	4212	42
TNBS at 30 min	60	1662	3183	1197	33	5422	7464	4023	44

[a] The lipid vesicles used were Deamer-Bangham vesicles formed from CFA$^-$ phospholipids extracted from strain FT17 grown in the presence of 1 μCi/mL of [1-^{14}C]acetate. The CFA synthase assay (final volume 0.1 mL) consisted of 40 mM sodium bicarbonate (pH 8.2), 25 μg of ^{14}C-labeled Deamer-Bangham vesicles, 0.4 mM SAM (0.125 μCi/μmol), and 25 μL of CFA synthase purified by method B. In experiment 1, after incubation at 37 °C for the times indicated, the reaction mixtures were diluted with 0.4 mL of 0.8 M sodium bicarbonate (which completely inhibits CFA synthase), followed by addition of 20 μg of carrier-unlabeled sonicated FT17 lipid vesicles and 1 mg of TNBS (50 μL of a 2% solution). After incubation at 22 °C for 30 min, the TNBS reaction was ended by addition of 0.1 mL of 8.5 N HCl, followed by lipid extraction and thin-layer chromatography as described under Materials and Methods. In experiment 2, 10 μL of a 2% TNBS solution was added directly to the CFA synthase assay mixture at either 0 or 30 min of incubation. After incubation at 30 °C for 30 min after TNBS addition, the reactions were stopped, and the lipids were extracted and chromatographed the same as in experiment 1.

To test this hypothesis, we used trinitrobenzenesulfonic acid (TNBS), a reagent which reacts with the amine groups of PE. Litman and co-workers (Litman, 1973, 1975; Roseman et al., 1975) have shown that under the proper conditions, single-walled phospholipid vesicles are impermeable to TNBS. Reaction of TNBS with PE to form TNBS–PE indicates that the modified PE molecule is located in the outer monolayer leaflet of the vesicle bilayer. Those PE molecules that do not react with TNBS are considered to be localized in the inner leaflet of the vesicle bilayer. TNBS–PE is separated from unmodified PE by thin-layer chromatography (see Materials and Methods).

The vesicles used in the TNBS experiments were made from unfractionated CFA$^-$ lipids by the ether evaporation method of Deamer & Bangham (1976). This method results in a population of fairly large (0.1-μm diameter) vesicles composed of a single lipid bilayer. These vesicles were used rather than sonicated vesicles to avoid the problems of interpretation that result from the very different radii of curvature of the inner and outer leaflets of small sonicated vesicles (Litman, 1973). Under the proper conditions, Deamer–Bangham vesicles made of unfractionated *E. coli* lipids were impermeable to TNBS [see Gordesky et al. (1975) for a discussion of the influences of reaction conditions on TNBS labeling]. Impermeability was shown by two types of experiments. First, only half of the total PE in the vesicles was converted to TNBS–PE during the first 30 min of reaction with TNBS (Figure 7A), whereas lysis of the vesicles with Triton X-100 (Litman, 1975) resulted in the conversion of all the PE to TNBS–PE (Figure 7A). In the second experiment, we trapped the tripeptide, triglycine, inside the vesicles by forming the vesicles in 0.2 M triglycine. The exogenous triglycine was removed by gel filtration, and TNBS was added to the triglycine-loaded vesicles. Reaction of TNBS with triglycine was monitored by the increase in absorbance of water-soluble material which had reacted with TNBS (see Materials and Methods). As shown in Figure 7B, the reaction of TNBS with triglycine was very slow in the intact vesicles. However, disruption of the vesicles with Triton X-100 resulted in a rapid increase in the rate of the reaction. The amount of triglycine trapped in the vesicles enabled us to calculate the internal volume of the *E. coli* lipid vesicles. Our value of 13 μL/μmol of phospholipid was very similar to that found by Deamer & Bangham (1976) for phosphatidylcholine vesicles (14 μL/μmol). This result indicated that

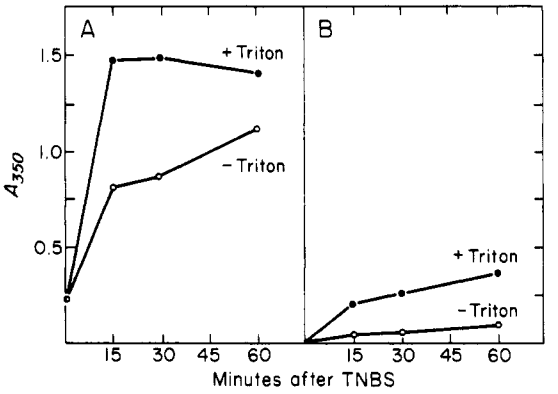

FIGURE 7: Reaction of triglycine-loaded Deamer–Bangham vesicles with TNBS. The vesicles were made according to Deamer & Bangham (1976) except that 0.8 M triglycine was present during vesicle formation. Exogenous triglycine was removed by chromatography on a column of Sephadex G-25 eluted with 0.2 M potassium phosphate buffer, pH 7.5. The TNBS reaction mixture (0.74-mL final volume) contained CFA$^-$ liposomes (75 μg), TNBS (0.8 mg), sodium bicarbonate (0.2 M; pH 8.2), and potassium phosphate (0.06 M) (from the vesicle preparation). Triton X-100 was added as indicated to 0.4%. After incubation at 22 °C for the times shown, the reactions were ended by addition of 0.2 mL of 8.5 N HCl, and the mixture was extracted by the method of Bligh & Dyer (1959). The phospholipids were in the $CHCl_3$ phase resulting from the extraction whereas the triglycine partitions quantitatively into the aqueous methanol phase. The absorbances at 350 nm were read for both phases.

the average diameter of our *E. coli* phospholipid vesicles was about 0.1 μm. This estimate was also in accord with the sedimentation properties of these vesicles (data not shown). Our vesicle preparations therefore appeared to be composed of fairly large, single bilayer vesicles which had half the PE molecules in each leaflet. Incubation of these vesicles with the standard CFA synthase reaction mixture resulted in a marked increase in the permeability of the vesicles to TNBS. However, the use of bicarbonate buffer and of low concentrations of SAM and protein (by omission of the SAH hydrolase preparation and use of highly purified CFA synthase) restored impermeability to TNBS. Using these modified assay conditions, we performed two types of experiments.

In the first type of experiment (Table VII, experiment 1), vesicles were made from phospholipids labeled with ^{14}C by growth of *E. coli* in the presence of [1-^{14}C]acetate. These

vesicles were incubated with CFA synthase and [*methyl*-^{3}H]SAM. After various periods of reaction, the reaction mixture was diluted, and carrier lipid vesicles (nonradioactive) and TNBS were added. Following completion of the TNBS reaction, the incubation mixture was extracted into chloroform–methanol and the lipids were separated by thin-layer chromatography. The resolved lipids were then assayed for both ^{14}C and ^{3}H. As shown in Table VII only half of the ^{14}C-labeled PE reacted with TNBS, thus indicating that TNBS did not enter the vesicles. However, about one-third of the ^{3}H label was found in PE which had not reacted with TNBS. If the vesicles were disrupted with Triton X-100 before the addition of TNBS, almost all of the PE (both ^{14}C and ^{3}H) reacted with TNBS. These results therefore indicate that a population of PE molecules in the vesicles is accessible to CFA synthase but is not accessible to TNBS.

In the second type of experiment (Table VII, experiment 2), TNBS was included in the enzyme reaction mixture. Although a slight leakage of TNBS into the vesicles was observed in these experiments, again one-third of the methylene groups was incorporated into PE molecules that were inaccessible to TNBS and thus (by definition) on the interior leaflet of the vesicle. The portion of cyclopropane-labeled PE which is inaccessible to TNBS does not change if CFA synthase activity was inactivated by addition of Nbs_2, an inhibitor of the reaction (data not shown).

Conclusions

CFA synthase from *E. coli* is an enzyme of about 90 000 daltons which is loosely bound to the inner membrane of the cell. The enzyme requires the presence of lipid vesicles for stability, binds to lipid vesicles, and can be appreciably purified by virtue of this association.

CFA synthase has several unusual properties. The enzyme binds to phospholipid vesicles and appears able to act on the phospholipid molecules of both leaflets of a phospholipid bilayer. This latter finding suggests that the enzyme penetrates into the bilayer. However, if enzyme activity requires penetration, penetration occurs equally well into phospholipids in either the ordered or the disordered phases. More definitive tests of the mechanism of CFA synthase must await purification of the protein to homogeneity.

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