Cyclopropane Fatty Acid Synthase of *Escherichia coli*: Deduced Amino Acid Sequence, Purification, and Studies of the Enzyme Active Site†

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ABSTRACT: Cyclopropane fatty acid (CFA) synthase of *Escherichia coli* catalyzes a modification of the acyl chains of phospholipid bilayers. We report (i) identification of the CFA synthase protein, (ii) overproduction (>600-fold) and purification to essential homogeneity of the enzyme, and (iii) the amino acid sequence of CFA synthase as deduced from the nucleotide sequence of the *cfa* gene. CFA synthase was overproduced by use of the T7 promoter/RNA polymerase system under closely defined conditions. The enzyme was readily purified by a two-step procedure requiring only ammonium sulfate fractionation and binding to phospholipid vesicles followed by flotation in sucrose density gradients. The deduced amino acid sequence predicts a protein of 43 913 Da (382 residues) that lacks long hydrophobic segments. The CFA synthase sequence has no significant similarity to known proteins except for sequences found in other enzymes that utilize *S*-adenosyl-L-methionine. We also report inhibitor studies of the enzyme active site.

Cyclopropane fatty acids (CFAs) have for several decades been known to occur in the lipids of many different eubacteria (Goldfine, 1982) although regulatory and physiological aspects of their formation have been most thoroughly studied in *Escherichia coli*. In recent years molecular cloning of a gene (*cfa*) that apparently encodes the *E. coli* CFA synthase allowed study of various physiological aspects of CFA synthesis (Grogan & Cronan, 1984a,b), such as the timing of CFA formation (which occurs during the onset of the stationary phase of growth), and of the biological function of CFAs, whose absence or overproduction has only minor physiological consequences in laboratory strains (Grogan & Cronan, 1984a, 1986). However, the lack of a purified source of CFA synthase has severely limited study of the unusual biochemical properties of this enzyme.

CFA synthesis proceeds by transfer of a methylene group from the activated methyl of *S*-adenosyl-L-methionine (S-AdoMet) to the cis double bond of an unsaturated fatty acid (UFA) chain, resulting in replacement of the double bond with a methylene bridge. In the reaction two of the three methyl protons (Pohl et al., 1963), the cis configuration of the acyl chain (Cronan, et al., 1974), and the vinyl protons of the UFA (Polachek et al., 1966) are retained. These findings severely limit the possible models for formation of the cyclopropane ring (e.g., cyclopropene intermediates are excluded) and the mechanism of cyclopropane ring formation remains unknown.

In *E. coli* the enzyme catalyzing the reaction, CFA synthase, is a soluble enzyme found in the cell cytoplasm (Taylor & Cronan, 1979) whose lipid substrate is not free UFA but rather phospholipids containing UFA (Zalkin et al., 1963; Cronan et al., 1974; Taylor & Cronan, 1979). This soluble enzyme thus has one substrate that is soluble (S-AdoMet) and a second that is insoluble (the phospholipid bilayer). The substrate double bond must be positioned 9–11 carbon units from the ester bond linking the UFA to the phospholipid "backbone" (Marinari et al., 1974) and thus is known to be located deep within the hydrophobic core of the bilayer structures of either phospholipid vesicles or the membranes of intact *E. coli* cells (Seelig & Seelig, 1980; Gally et al., 1979). CFA synthase binds only to phospholipid vesicles made of phospholipids which contain either unsaturated or cyclopropane acyl chains (Taylor & Cronan, 1979), indicating that a specific protein–lipid interaction is responsible for the recognition and binding of substrate bilayers. Other data suggest that CFA synthase somehow gains access to both the inner and outer leaflets of intact phospholipid vesicles (Taylor & Cronan, 1979).

Unfortunately, detailed analysis of these protein–lipid interactions has been severely hampered by the extreme lability of CFA synthases obtained from a variety of bacteria (Chung & Law, 1964; Crowfoot & Hunt, 1971; Taylor & Cronan, 1979). For example, in the absence of phospholipid, *E. coli* CFA synthase has a half-life of only 16 h at 4 °C and of only several minutes at 37 °C. This lability had precluded purification of any CFA synthase to homogeneity. Indeed, only the stabilization of the *E. coli* enzyme by substrate phospholipid vesicles allows routine assay of the enzyme (Taylor & Cronan, 1979). The isolation of the *cfa* gene allowed overproduction of CFA synthase by use of expression vectors (Grogan & Cronan, 1986), but the degree of enzyme overproduction was erratic and seemed to depend on ill-defined physiological and genetic parameters (Grogan, 1985). In this paper we report that expression of CFA synthase from a phage T7 promoter results in consistent and massive overproduction of this enzyme. We also report the nucleotide sequence of the *cfa* gene, the deduced amino acid sequence of the protein, and studies of the enzyme active site.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* BL21 (λDE3) (Studier & Moffatt, 1986) was used to express the *cfa* gene in the T7 polymerase expression system, and strain CSR603 (Sancar et al., 1979) was used for maxicell analysis. The plasmids used were pGI22 (Grogan & Cronan, 1986), pGI6, and pGI5 (Grogan & Cronan, 1984a). Media

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and growth conditions were those previously described (Grogan & Cronan, 1984a).

Plasmid Constructions. The 2.0-kbp *Cla*I fragment of pGI22 containing the entire *cfa* gene was purified by gel electrophoresis, and the recessed ends were filled in with the Klenow fragment of DNA polymerase I. The resulting blunt-ended fragment was inserted into the *Eco*RV site of pGEM-5Zf(+) (Promega) in both orientations relative to the *lacZ* promoter to form plasmids pAYW19 and pAYW20. To obtain the expression plasmid pAYW58, the *Acc*I–*Hin*dII fragment containing the Shine–Dalgarno sequence and entire *cfa* coding region was excised from pAYW19. The single-stranded end of the fragment was filled by Klenow fragment, and the fragment was then ligated into pET3 (Rosenberg et al., 1987) which had been digested with *Bam*HI and filled in with Klenow fragment.

Analysis of Clone-Specific Proteins. Various *cfa*$^+$ and *cfa*$^-$ plasmids introduced into strain CSR603 were analyzed for protein product by the maxicell procedures of Sancar et al. (1979). The in vitro transcription–translation reactions were carried out with S30 extracts obtained from Promega. Both systems used [^{35}S]methionine incorporation to monitor protein synthesis. Radiolabeled proteins were analyzed on 10% SDS–polyacrylamide gels by the procedure of Laemmli (1970). Protein standards were radioactively labeled by dissolving 5 mg of protein in 0.5 mL of $NaBO_4$ (50 mM, pH 9) and adding 10 μCi (0.17 μmol) of [^{14}C]ethyl acetimidate (New England Nuclear) followed by incubation for several hours at 25 °C. Under these conditions, less than one [^{14}C]ethyl acetimidate molecule (on average) was incorporated per protein molecule. In later work commercially labeled standards (Bethesda Research Laboratories) were used.

DNA Sequencing. Plasmid pAYW19 and pAYW20 were submitted to exonuclease III–S1 nuclease digestion (Hoheisel & Pohl, 1986), and a series of unidirectional deletions were sequenced as double-stranded DNA by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase reagents from U.S. Biochemicals.

Overproduction and Purification of CFA Synthase. E. coli BL21 (λDE3) was transformed with plasmid pAYW58. Transformants were grown in 2× YT medium containing 0.1% glucose and 100 μg/mL ampicillin. Expression of the *cfa* gene was induced at midlog phase by adding IPTG to a final concentration of 40 μM. After being shaken at 37 °C for another 2 h, the cells were harvested by centrifugation and washed with 0.1 M potassium phosphate buffer, pH 7.5.

For purification of CFA synthase, washed cells were suspended in 0.1 M potassium phosphate buffer, pH 7.5. The cells were disrupted by two passages through a French pressure cell at 11 000 psi. The resulting lysate was centrifuged at 150000*g* for 2 h. This and all subsequent purification steps were performed at 0–4 °C. Solid ammonium sulfate was added to the centrifugation supernatant to 40% saturation. After centrifugation, the protein pellet was resuspended in 40% saturated ammonium sulfate solution (in 50 mM potassium phosphate buffer, pH 7.5) and stirred for 2 h. The precipitate was collected by centrifugation at 10000*g* for 15 min and dissolved in the same phosphate buffer. Residual ammonium sulfate was removed by gel filtration on Sephadex G-25 in the same phosphate buffer. Liposome flotation of CFA synthase was carried out by the method of Taylor and Cronan (1979) except a single flotation with UFA-containing liposomes was used and 0.15 M NaCl was included in all solutions. After centrifugation, the lipid layer was removed and mixed with sufficient sucrose and NaCl to give final concentrations of 30% (w/v) and 0.2 M, respectively. This mixture was overlaid with layers of 25% and 20% sucrose in the presence of 0.2 M NaCl and the phosphate buffer and centrifuged at 80000*g* for 2 h as described by Taylor and Cronan (1979). The vesicle-bound synthase was removed and stored at –20 °C.

CFA Synthase Assay and Protein Determination. The CFA synthase activity was assayed at 37 °C as described by Taylor and Cronan (1979). One unit of CFA synthase activity is defined as 1 pmol of CFA formed per minute at 37 °C. The protein content of cell extracts or enzyme solutions was determined by the biuret method (Gornall et al., 1949).

Phospholipid Isolation and Fatty Acid Analysis. Preparation of phospholipids and liposomes were obtained as described previously (Taylor & Cronan, 1979). Fatty acid methyl esters were prepared from membrane phospholipids and analyzed by gas chromatography on a DB-23 polysilicone capillary column (J&W Scientific).

Inactivation by N-Alkylmaleimides. N-Alkylmaleimides, synthesized by the method of Schwartz and Lenner (1974), were purified by chromatography over silica gel and repeated crystallization from diethyl ether. Purity was determined to be 96–97%, based on ultraviolet adsorbance (Gregory, 1955). Ethanolic solutions of the *N*-alkylmaleimides (2.0 μL) were added to duplicate reaction mixtures containing partially purified CFA synthase, 50 mM potassium phosphate (pH 6.8), and 1 mM EDTA in a final volume of 300 μL at 26 °C. Samples (40 μL) were withdrawn from each mixture at various times after maleimide addition and immediately transferred to CFA synthase assay mixture at 0 °C containing reduced glutathione at a final concentration of 10 mM to quench the reaction. The assay tubes were incubated for 50 min at 37 °C and the remaining CFA synthase activity was determined.

To prepare the partially purified CFA synthase for this study, extracts of plasmid-amplified strains were freed of endogenous membrane lipids by centrifugation at 300000*g* for 90 min. The supernatant was concentrated to 10% of its volume by lyophilization and then chromatographed in 50 mM potassium phosphate, pH 7.5, over a small column of Bio-Gel P-30 at 4 °C. Fractions corresponding to the void volumn were pooled, portioned into aliquots, and stored at –70 °C.

Chemicals and Reagents. Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. S-AdoMet was from Sigma Chemical Co. [*methyl*-^{3}H]-S-AdoMet was from New England Nuclear Corp. Isopropyl thiogalactoside was obtained from Bethesda Research Laboratories. Sinefungin and A9145C were obtained from Calbiochem and Chemical Dynamics Corp., respectively.

RESULTS

Identification of the cfa Gene Product. Our approach to overproduction of CFA synthase activity was to first concentrate on overproduction of the protein per se and then optimize enzyme activity. We, therefore, identified the protein encoded by the *cfa* gene in order that we could assay its production.

We used previously constructed plasmid and phage λ clones (Grogan & Cronan, 1983, 1984a, 1986) that carried a functional *cfa* gene and overproduced CFA synthase and identified the *cfa* gene product as a protein of ca. 40 kDa on SDS–polyacrylamide gels. Proteins encoded by *cfa*$^+$ and *cfa*$^-$ (deletion) plasmids were labeled in vivo using the maxicell procedure of Sancar et al. (1979) (Figure 1) or the in vitro

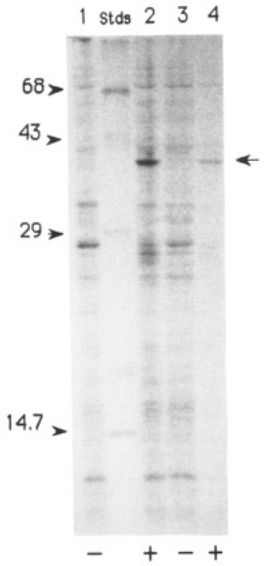

FIGURE 1: Radiochemical labeling of CFA synthase in vivo. Proteins encoded by *cfa*$^+$ and *cfa*$^-$ plasmids were pulse-labeled with L-[^{35}S]-methionine in maxicells as described in Materials and Methods. An autoradiograph (3-day exposure) is shown. Molecular weight standards (bovine serum albumin, ovalbumin, lactate dehydrogenase, and lysozyme) are indicated at margin; CFA phenotypes (overproduced enzyme activity) appear below each lane. Lane 1, pBR322; lane 2, pGI6; lane 3, pGI15; lane 4, pGI13. The *cfa*$^-$ plasmids are deleted derivatives of the *cfa*$^+$ plasmid. For detailed descriptions of the plasmids, see Grogan and Cronan (1984a).

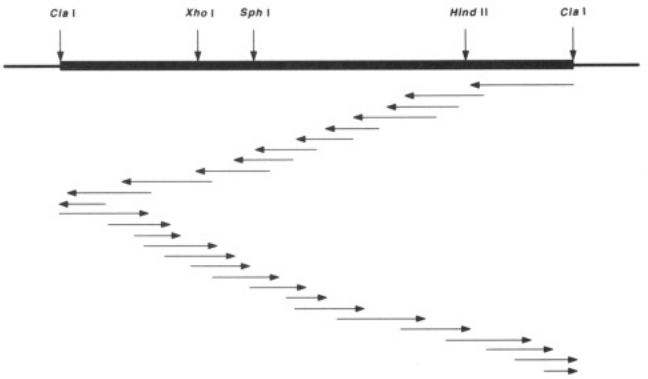

FIGURE 2: Sequencing strategy. The *cfa* gene is located in the *Cla*I–*Hin*dII region. The direction of translation is from left to right and the nested deletions in this direction were derived from pAYW20. The other plasmids were derived from pAYW19.

transcription–translation procedure of Chen and Zubay (1983) (data not shown). Both labeling protocols showed a protein of ca. 40 kDa to be present in plasmids containing a functional *cfa* gene but absent in plasmids lacking a functional *cfa* gene due to engineered deletions within the *E. coli* chromosomal DNA insert (Figure 1). An identical correlation was also found for a 40-kDa protein encoded by several independent *cfa*-containing phages labeled following infection of UV-irradiated cells (Grogan, 1985). An indication that the *cfa* gene was the structural gene encoding CFA synthase (rather than a regulatory gene) was the observation that the 40-kDa protein synthesized in vitro bound to the membrane vesicles present in the transcription–translation extracts (data not shown). CFA synthase is known to bind to *E. coli* membrane vesicles (Taylor & Cronan, 1979), whereas a regulatory protein is expected to lack this property. Thus, it seemed that the 40-kDa protein catalyzed the CFA synthase reaction. It should be noted that the *cfa* gene had been localized to a DNA segment of <1500 bp and, thus, was known to encode a protein no larger than 55 kDa (Grogan & Cronan, 1986).

Nucleotide Sequence of the cfa Gene. To further define the size of the CFA synthase protein and to facilitate the construction of new expression vectors, we determined the nucleotide sequence of the *cfa* gene. The *Cla*I fragment containing the entire gene (Grogan & Cronan, 1986) was sequenced as described in Materials and Methods (Figure 2). Open reading frame (ORF) analysis showed that only one major ORF having a good potential ribosome-binding site was present within the *Cla*I–*Hin*dII DNA segment. The complete DNA sequence and deduced amino acid sequence of the fragment are shown in Figure 3. The ORF predicts a protein of 382 amino acids and p*I* 5.83. The predicted mass of the protein, 43 913 Da, is in reasonable agreement with the 40-kDa values obtained by SDS–PAGE for the labeled band seen in maxicell analysis of *cfa* plasmids and for the the purified enzyme (see below). Further indications that this ORF encoded CFA synthase were the following observations: (i) The N-terminal sequence obtained by automated Edman degradation of the purified protein (see below) matched the deduced sequence. (ii) The direction of *cfa* transcription and the translational reading frame previously established by the construction of a plasmid-borne *cfa–lacZ* fusion (Grogan & Cronan, 1986) are identical to those predicted by the sequence. (iii) Deletions of the 3′-end of the DNA segment encoding this ORF resulted in the loss of the 40-kDa protein and appearance of suitably truncated products, whereas deletions of the 5′-region upstream of the ATG start codon resulted in the loss of 40-kDa protein (data not shown). (iv) Expression of the coding sequence and upstream ribosome-binding site in the T7 transcription vector pET3 resulted in overproduction of the 40-kDa gene product and a dramatic increase in the activity of CFA synthase in vivo and in vitro (see below).

Overexpression and Purification of CFA Synthase. CFA synthase was produced in *E. coli* strain BL21 (λDE3) transformed with plasmid pAYW58 carrying the *cfa* gene under control of a T7 promoter. Expression of the T7 promoter occurred upon induction of the synthesis of T7 RNA polymerase which in turn is under control of a *lac* promoter [and hence induced by isopropyl thiogalactoside (IPTG)]. In our first experiments we found that cells carrying the expression plasmid produced more CFA synthase activity in the absence of IPTG, the T7 polymerase inducer, than in the presence of IPTG (data not shown). This unexpected result was traced to formation of inactive, insoluble aggregates (inclusion bodies) of the overproduced CFA synthase protein when cells were maximally induced by high concentrations of IPTG (Figure 4). Although purification of inclusion bodies followed by protein solubilization and careful refolding has given effective purification of other enzymes, this approach was precluded by the extreme lability of CFA synthase in the absence of phospholipids. We, therefore, sought to produce the CFA synthase protein in a soluble form. Various growth conditions and IPTG concentrations were tested for expression of CFA synthase in soluble and active form. The optimal conditions for expression of CFA synthase were found when cells were grown in rich broth containing glucose at 37 °C and T7 RNA polymerase was induced by adding IPTG to a final concentration of 40 μM. Under these conditions the specific activity of CFA synthase was increased 650-fold over a strain lacking the cloned CFA synthase gene and most of the synthase protein was soluble and highly active (Figure 4). The overproduced CFA synthase in strain BL21 (λDE3, pAYW58) was also shown to be functional in vivo since the unsaturated fatty acid (UFA) moieties of log-phase cell phospholipids were quan-

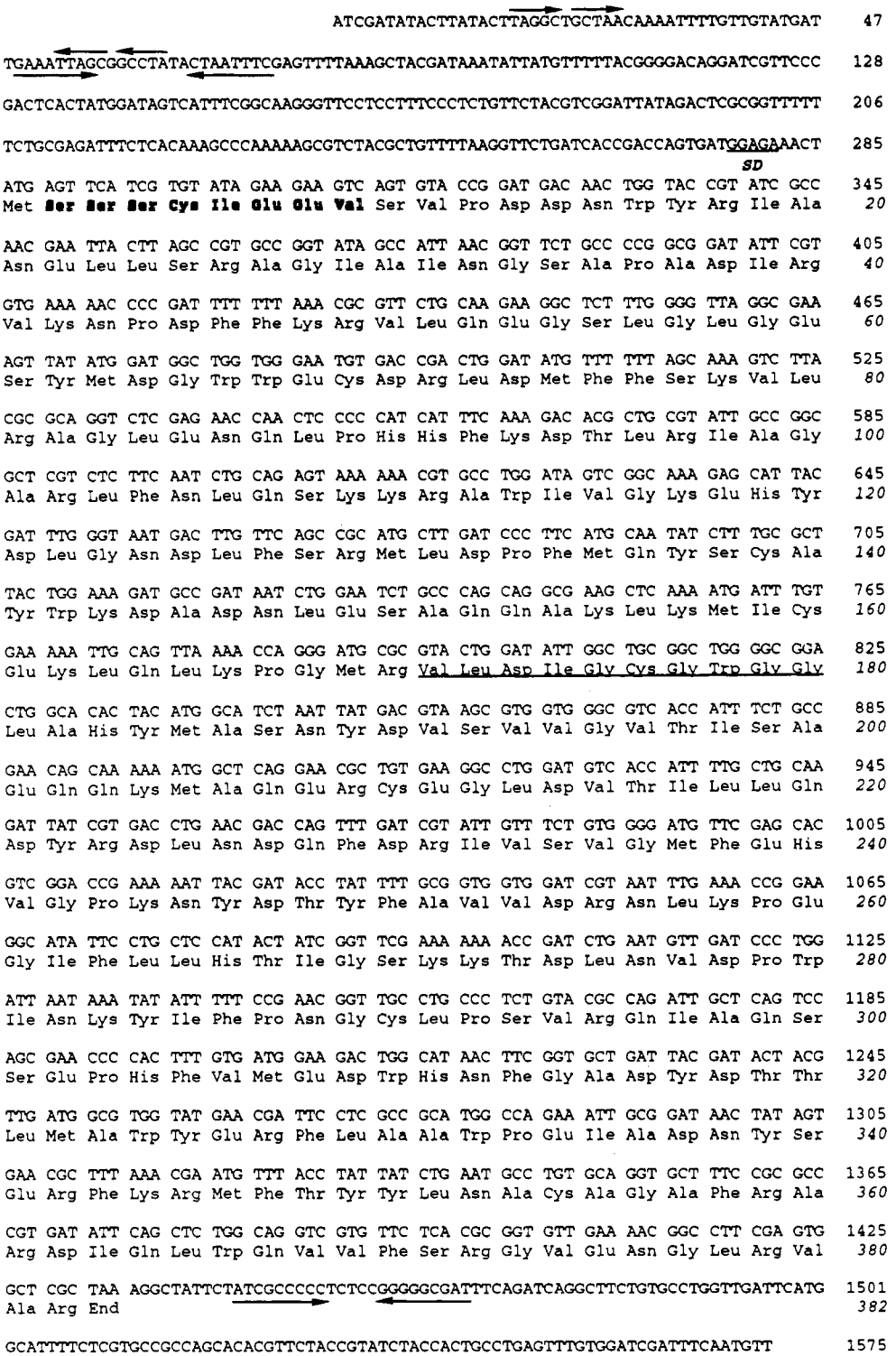

FIGURE 3: Nucleotide sequence of the *cfa* gene and deduced amino acid sequence of CFA synthase. The sequence determined by N-terminal sequencing is shown in boldface type. The Shine–Dalgarno (SD) ribosome-binding sequence and putative S-AdoMet binding site are underlined. Inverted repeat sequences are indicated by arrows. The sequence downstream of the *cfa* gene is that of the phage λ *git* gene derived from the λ vector used in earlier gene mapping experiments (Grogan & Cronan, 1983).

titatively converted to their cyclopropane derivatives (Table I), a process that requires overproduction of CFA synthase activity (Grogan & Cronan, 1984a). Production of large amounts of CFA synthase was critically dependent on the conditions of cell growth (Figure 4). For example, lowering the growth temperature to 30 °C resulted in little or no induction of the enzyme upon IPTG addition.

Although expression of the *cfa* gene from the T7 promoter by T7 RNA polymerase dramatically increased the amount of CFA synthase present in *E. coli*, the lability of the enzyme still precluded purification by conventional methods. For example, in the absence of phospholipid, dialysis at 4 °C for 5 h caused 95% loss of enzyme activity, and during FPLC (Pharmacia) on a Mono-Q column, the enzyme activity was completely lost in a 30-min run at room temperature (details not shown). Thus, the flotation technique of Taylor and Cronan (1979), the most successful technique previously applied to unamplified strains, was adopted.

The enzyme was first purified by ammonium sulfate fractionation. We found that the overproduced CFA synthase

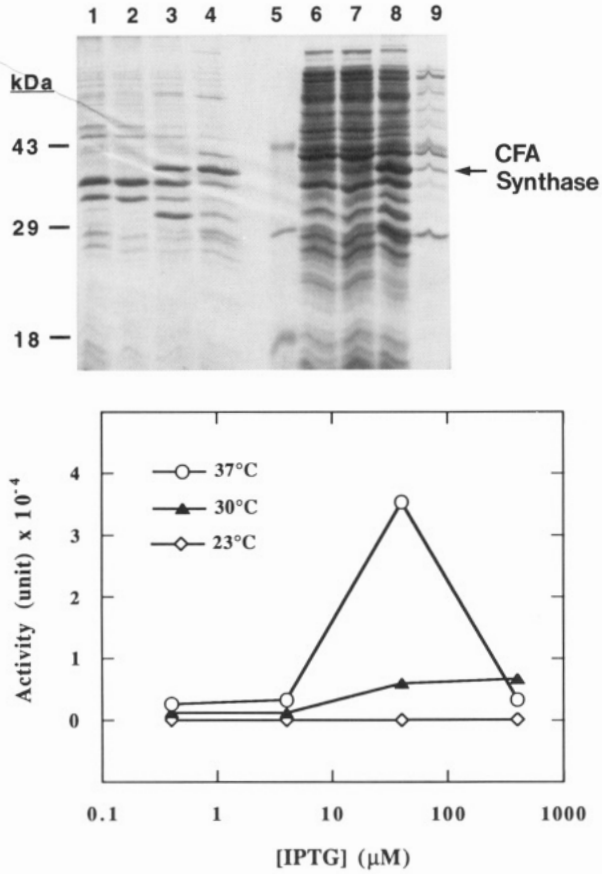

FIGURE 4: Production of CFA synthase. *E. coli* strain BL21 (λDE3, pAYW58) was grown in 2× YT containing 0.1% glucose and 100 μg/mL ampicillin at 37 °C. The culture was divided among several flasks when the OD_{600} reached 0.6 and various concentrations of IPTG were added. After shaking at 23, 30, or 37 °C for 2 h, 3-mL samples of each culture were harvested and the cells were washed with 0.1 M potassium phosphate buffer, pH 7.5. Cell pellets were lysed by lysozyme and repeated freezing and thawing. After centrifugation (27000*g* for 1 h), the supernatants and pellets were analyzed for the production of CFA synthase protein and activity. The arrow marks the CFA synthase protein band. (Top panel) Supernatants (lanes 6–9) and pellets (lanes 1–4) of cell lysates induced at 37 °C were analyzed on a 15% SDS–polyacrylamide gel. The gel was stained with Comassie blue after electrophoresis. CFA synthase was induced by adding IPTG to 0.4 μM (lanes 1 and 6), 4 μM (lanes 2 and 5), 40 μM (lanes 3 and 8), or 400 μM (lanes 4 and 9). Lane 5, molecular weight standards. The small amount of protein evident in lane 9 can be attributed to the inhibition of bulk protein synthesis seen after maximal induction of T7 RNA polymerase (Studier & Moffatt, 1986). (Bottom panel) Enzyme activities of CFA synthase in the supernatants of lysates of cells exposed to the various induction conditions.

was much more efficiently precipitated by ammonium sulfate than the activity present in extracts of nonamplified cells. Thus, precipitation gave a very significant purification with only ca. 30% loss in activity (no activity was lost in small-scale experiments), providing that the residual salt in the precipitate was removed rapidly (gel filtration) and liposomes were promptly added to stabilize the enzyme. The flotation technique previously used (Taylor & Cronan, 1979) could be simplified due to the greatly increased beginning specific activity obtained from the new expression system and the improved salt fractionation. We also performed the flotation with UFA-containing liposomes in the presence of 0.15 M NaCl to decrease nonspecific binding of proteins to the phospholipid vesicles. A final washing step, flotation of the vesicle-bound enzyme in the presence of 0.2 M NaCl, was introduced to remove remaining impurities. The overall recovery of CFA synthase activity was about 10–25% of that present in the crude extract (depending on the amount of starting material used) (Table II). Some large-scale purifications gave disappointing results (low yields and poor purity) for unknown reasons (presumably inadvertent inactivation of this labile enzyme). Quantitative determination of the final extent of purification was problematic. The very high concentrations (ca. 100 mg/mL) of phospholipids present in the final fractions precluded direct protein assays and attempts to remove of the phospholipid without loss of protein gave erratic results. We, therefore, determined the final protein concentrations by amino acid analysis of acid-hydrolyzed samples of the protein–phospholipid complex. Figure 5 shows SDS–polyacrylamide gel electrophoretic analysis of the course of CFA synthase purification. As judged from Coomassie blue staining, the purity is more than 95%; only a single major band was seen on these gels. The purified enzyme was stored at −20 °C in the presence of phospholipid. No significant loss of enzyme activity occurred during 2 months of storage.

Inhibitors of *Catalysis*. Several compounds which covalently modify sulfhydryl groups, including *N*-ethylmaleimide, were found to inactivate CFA synthase (Taylor, 1977). We have used this inactivation to probe the environment of the essential sulfhydryl group. Our approach was that of Anderson and co-workers (Anderson & Vacini, 1970; Anderson et al., 1970; Fonda & Anderson, 1969), who showed that enzymes having essential sulfhydryl groups in hydrophobic environments (such as the hydrophobic cleft of papain) are more readily inactivated by long-chain alkylmaleimides than by *N*-ethylmaleimide. Hydrophobic interactions between the enzyme and the long-chain alkyl group are thought to concentrate the maleimide in the vicinity of essential sulfhydryl group, thus resulting in increased rates of enzyme inactivation with increased chain length.

Lipid-free, partially purified CFA synthase preparations were treated with *N*-ethyl-, *N*-butyl-, *N*-pentyl-, *N*-hexyl-, or *N*-heptylmaleimide and the rates of inactivation were measured (Figure 6). Removal of endogenous lipid was necessary to demonstrate full sensitivity to these reagents. Under these conditions the kinetics of inactivation were pseudo-first-order and quite rapid compared to those of other enzymes analyzed in this fashion (Anderson et al., 1970; Fonda & Anderson, 1969). The pseudo-first-order rate constant, k_1, for each homolog was approximately proportional to inhibitor concentration in the range employed, enabling estimation of second-order rate constants (k_2). Except for *N*-ethylmaleimide, the apparent k_2 increased exponentially with increasing length of the alkyl chain, as expected for a purely hydrophobic interaction (Figure 6, inset). Thus, our results are those characteristic of reversible binding of the inhibitor in a hydrophobic pocket or cleft followed by nucleophilic attack of the localized maleimide by the neighboring enzyme-bound sulfhydryl group, resulting in irreversible inactivation of enzyme (Anderson & Vasini, 1970).

Additional properties of this hydrophobic site were observed by adding substrates to the inactivation mixtures. The soluble substrate, S-AdoMet, failed to protect CFA synthase against inactivation by *N*-hexylmaleimide (Table III), even at concentrations far exceeding the apparent K_m of 90 μM (Taylor & Cronan, 1979). In contrast, sonicated liposomes of UFA-containing phospholipids gave considerable protection. Half-maximal protection was obtained at approximately the concentration (50–80 μg/mL) resulting in half-maximal enzyme activity (Taylor & Cronan, 1979). Liposomes made of CFA-containing phospholipids, which are known to bind and stabilize the enzyme (Taylor & Cronan, 1979) also

Table I: Fatty Acid Compositions of Cellular Phospholipids

plasmid[a]	IPTG	methyl esters (wt %) C14:0	C16:0	C16:1	C17Δ	C18:1	C19Δ	% conversion to CFA[b] C16:1	C18:1
pET3	–	2.36	28.45	23.21	7.48	34.67	2.05	47.4	10.7
	+	2.07	30.94	18.91	13.07	31.76	3.23	40.9	9.2
pAYW58	–	2.48	39.20	<0.5	22.05	19.67	16.27	100	45.27
	+	2.38	35.48	<0.5	22.88	2.96	35.68	100	92.3

[a] The host strain was *E. coli* BL21 (λDE3). Cultures were grown in 2× YT medium containing 0.1% glucose and 0.1 mg/mL ampicillin at 37 °C. The culture was divided into two flasks when the turbidity reached 75 Klett units (about 2×10^8 cells/mL). IPTG was added to one flask at a final concentration of 40 μM. Cells were harvested 60 min after IPTG induction. [b] Percent conversion was calculated by the formula [% CFA/(% CFA + % UFA)] × 100.

Table II: Purification of CFA Synthase[a]

purification step	tot. act. (units)	tot. protein (mg)	sp act. (units/mg)	purification (*x*-fold)	recovery (%)
crude extract	1.95×10^6	152[b]	1.28×10^4	1	100
$(NH_4)_2SO_4$ fractionation	1.36×10^6	28.5[b]	4.77×10^4	3.7	69.7
flotation	3.74×10^5				19.2
washing	3.50×10^5	0.85[c]	4.12×10^5	32.2	17.9

[a] Purification from cells of a 500-mL culture of IPTG-induced *E. coli* BL21 (λDE3, pAYW58). [b] Protein concentrations were determined by the biuret method. [c] Protein concentrations were determined by amino acid analysis of acid-hydrolyzed samples of the protein–phospholipid complex.

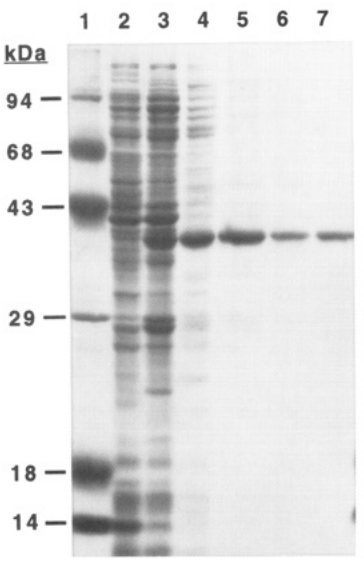

FIGURE 5: Purification of CFA synthase. Protein samples from various stages were separated on a 12.5% SDS–polyacrylamide gel. The gel was stained with Coomassie blue after electrophoresis. Lane 1, molecular weight standards; lane 2, crude extract of the cloning vector containing strain *E. coli* BL21 (λDE3, pET3) harvested after IPTG induction for 2 h; lane 3, crude extract of the overproduction strain, *E. coli* BL21 (λDE3, pAYW58); lane 4, ammonium sulfate (0–40%) precipitate; lane 5, liposome flotation of the ammonium sulfate purified enzyme; lanes 6 and 7, samples of the final flotation washing step described in Materials and Methods.

protected against *N*-hexylmaleimide, but liposomes made of SFA-containing phospholipids failed to protect (Table III). This latter observation demonstrated that the protection observed by liposomes was not a trivial effect due to a passive sequestering of *N*-hexylmaleimide by the liposomes. The essential sulfhydryl of CFA synthase thus resides in or near a hydrophobic site which binds phospholipid in the same discriminatory manner as the enzyme, implicating this sulfhydryl group in the catalytic mechanism.

The soluble product of CFA synthase, *S*-adenosyl-L-homocysteine, represents another class of compounds inhibiting

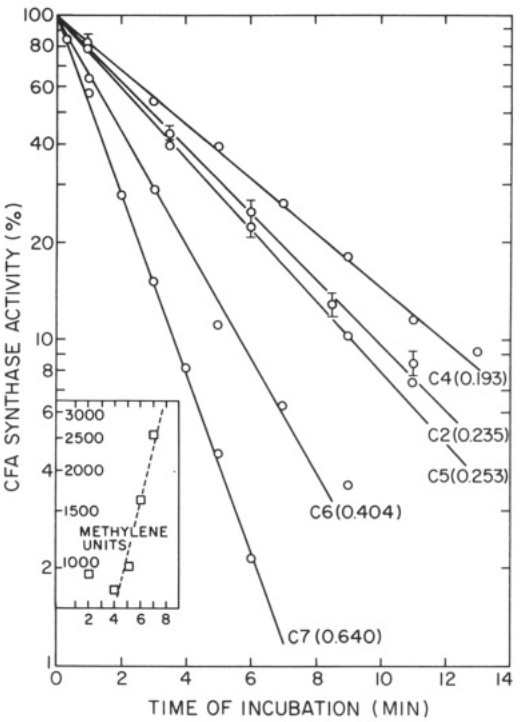

FIGURE 6: Inactivation of CFA synthase by *N*-alkylmaleimides. CFA synthase was incubated at 26 °C with 250 μM *N*-alkylmaleimide of various chain lengths as described in Materials and Methods. Pseudo-first-order rate constants (K_1, min^{-1}) calculated from the slopes of the activity curves are included in parenthese. Error bars indicate the range of values given by two independent trials. The inset graph shows the apparent second-order rate constants (K_2, M^{-1}, min^{-1}) plotted as a function of alkyl chain length.

the enzyme. Product inhibition is a common feature of methyltransferases, and certain structural analogs of S-AdoMet and antibiotic compounds have been isolated which are particularly effective competitive inhibitors of such enzymes. We tested the ability of two of these compounds, sinefungin and A9145C, to inhibit highly purified CFA synthase of *E. coli* K-12. The latter compound was found to be particularly effective (Figure 7); on the basis of the concentrations giving half-maximal inhibition, sinefungin was 2–3 times more inhibitory than *S*-adenosyl-L-homocysteine (Taylor & Cronan, 1979) whereas compound A9145C was about 200 times more inhibitory (apparent $K_i = 1$ μM). These relative potencies correspond to those observed for other S-AdoMet-dependent enzymes.

DISCUSSION

Although CFA synthase was discovered almost 30 years ago (Zalkin et al., 1963), the lack of purified enzyme has precluded detailed study. Thus, little is known concerning the topology and mechanism of this reaction. We have finally succeeded in obtaining large quantities of purified CFA

Table III: Protection of CFA Synthase from *N*-Hexylmaleimide Inhibition[a]

addition	concn (μM or μg/mL)	relative inactivation	% protection
S-adenosyl-L-methionine	0	1.00	0
	30	1.00	0
	100	0.84	16
	300	0.99	1
	1000	0.86	14
UFA vesicles	0	1.00	0
	3	0.83	17
	10	0.65	35
	20	0.62	38
	50	0.58	42
	200	0.54	46
	500	0.46	54
	1000	0.40	60
	2500	0.28	72
CFA vesicles	10	0.82	18
	50	0.65	35
	500	0.45	55
SFA vesicles	10	0.94	6
	50	1.02	0
	500	0.99	1

[a] Partially purified CFA synthase was inactivated by *N*-hexylmaleimide as described in Figure 6. Additions were made to the indicated final concentrations prior to inhibitor addition. S-AdoMet concentrations are given as micromolar, whereas vesicle concentrations are given as micrograms per milliliter of phospholipid. Relative inactivation equals the slope of the inactivation curve normalized to that of the control (no additions) of each set. UFA, CFA, and SFA vesicles denote vesicles composed of UFA, CFA, or saturated fatty acids (SFA), respectively (Taylor & Cronan, 1979).

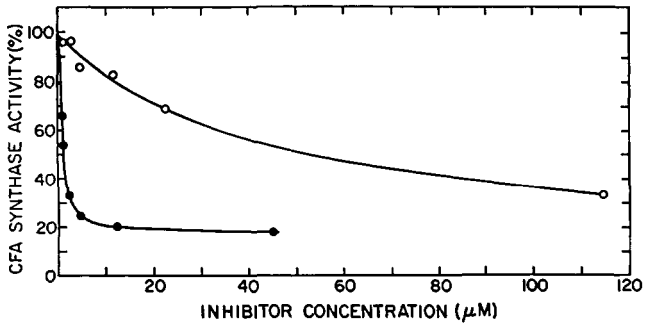

FIGURE 7: Inhibition of CFA synthase by analogs of S-AdoMet. Duplicate samples of CFA synthase purified through the flotatio step were assayed in the presence of either sinefungin (O) or A9145 (●).

synthase which will allow detailed study of this novel enzyme. Two aspects of our purification protocol deserve mention. First, the induction of enzyme synthesis had to be carefully controlled to prevent formation of intracellular protein aggregates (inclusion bodies). We reasoned that if aggregation results from collision of partially folded CFA synthase molecules, then a decreased rate of synthesis should lower the concentration of such molecules and prevent aggregation. We therefore decreased the rate of *cfa* gene transcription by decreasing the expression of phage T7 RNA polymerase. We also expected that decreased growth temperature should decrease aggregation, since lower temperatures decrease hydrophobic interactions. However, induction at 30 °C gave no significant overproduction of CFA synthase protein. Second, we found that the overproduced soluble protein was purified more efficiently by ammonium sulfate precipitation than was the enzyme in nonamplified extracts (Taylor & Cronan, 1979; Taylor et al., 1981). This phenomenon of increased ammonium sulfate precipitation of an overproduced protein is not unique to CFA synthase. It was first observed

															Accession #	Enzyme
M	R	V	L	D	I	G	C	G	W	G	G	L	A	H	M98330	CFA Synthase
F	L	N	A	I	F	P	N	G	A	G	Y	C	M	G	M21154	DC
A	K	A	L	D	V	G	S	G	S	G	I	L	T	A	M60320	CMT
P	E	V	S	V	L	D	V	G	C	G	G	G	I	L	M73270	DBMT
G	P	A	A	S	E	T	C	G	D	G	G	R	G	E	J02671	HOMT
P	L	I	C	D	L	G	G	G	S	G	A	L	A	K	M81862	HOMT
S	T	L	V	D	V	G	G	G	V	G	A	T	L	H	M73235	LOMT
A	D	R	E	K	T	R	V	G	S	G	G	H	W	S	M16987	PMT
S	Q	H	A	C	L	I	E	G	K	G	E	C	W	Q	J03727	PNMT
D	V	H	R	P	Q	P	L	G	A	G	G	L	A	P	M14318	PNMT
K	G	G	D	P	F	V	F	G	R	G	G	E	E	I	M62874	UMT
K	G	G	D	P	F	V	F	G	R	G	G	E	E	A	M62881	UMT
D	R	A	D	P	L	E	Q	G	A	G	D	Q	G	L	K02129	Syn
K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	M55077	Syn
K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	M61882	Syn
L	R	V	M	S	L	F	S	G	I	G	A	F	E	A	K02124	NAMT
L	R	V	M	S	L	F	S	G	I	G	A	F	E	A	M13488	NAMT
R	M	V	E	L	G	F	S	G	V	G	G	S	L	Y	M19269	NAMT
H	V	F	M	D	L	F	S	G	T	G	I	V	G	E	M28828	NAMT
E	G	H	R	L	G	K	P	G	L	G	G	S	S	G	M31767	NAMT
D	T	V	L	D	I	G	A	G	K	G	F	L	T	V	M62487	NAMT
T	L	T	S	Q	S	V	G	G	L	G	G	Q	T	S	M72412	NAMT
R	T	V	D	D	R	P	Y	G	G	G	P	G	M	L	X01818	NAMT
T	L	T	S	Q	S	V	G	G	L	G	G	Q	T	S	X05242	NAMT
M	K	I	I	S	L	F	S	G	C	G	G	L	D	L	X06965	NAMT
F	R	F	I	D	L	F	A	G	I	G	G	I	R	R	X13330	NAMT
D	L	V	V	D	I	F	G	G	S	N	T	T	G	L	X13778	NAMT
S	T	V	L	D	F	F	A	G	S	G	V	T	A	R	X16456	NAMT
D	L	V	I	D	P	F	C	G	S	G	S	T	L	H	X17111	NAMT
L	T	F	I	D	L	F	A	G	I	G	G	I	R	L	X51515	NAMT
S	A	S	R	G	T	K	A	G	G	G	G	R	P	G	X51891	NAMT
Y	N	V	F	E	T	F	A	G	A	G	G	L	A	L	X53096	NAMT
K	V	F	C	D	L	F	A	G	T	G	I	V	G	R	X54485	NAMT
L	K	F	I	D	L	F	A	G	I	G	G	M	R	L	X55138	NAMT
F	R	F	I	D	L	F	A	G	I	G	G	F	R	L	X55139	NAMT
M	K	T	I	D	L	F	A	G	C	G	G	M	S	L	X55140	NAMT
G	A	V	I	D	L	F	C	G	V	G	G	L	T	H	X55141	NAMT
F	R	F	I	D	L	F	A	G	I	G	G	F	R	L	X55142	NAMT
M	K	T	I	D	L	F	A	G	C	G	G	M	S	L	X55143	NAMT
D	L	V	L	D	C	F	M	G	S	G	T	T	A	I	X56977	NAMT

FIGURE 8: Common region of *S*-adenosyl-L-methionine-utilizing enzymes. All sequences except CFA synthase were obtained from the Genbank and EMBL databases using the String Search and Translation programs. The motif search was done using the Motif program of Smith et al. (1990). DC, S-AdoMet decarboxylase; CMT, protein carboxyl methyltransferase; DBMT, 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase; HOMT, hydroxyindol *O*-methyltransferase; LOMT, lignin *O*-methyltransferase; PMT, phospholipid methyltransferase; PNMT, phenylethanolamine *N*-methyltransferase; UMT, S-AdoMet–uroporphyrinogen III methyltransferase; Syn, S-AdoMet synthetase; NAMT, nucleic acid specific methyltransferase.

by K. Reed of this laboratory in her studies of a protein of lipoic acid metabolism and has also been observed by Pognonec et al. (1991) for several recombinant eucaryotic proteins synthesized in *E. coli*. This increased susceptibility to precipitation seems due to a self-association of the overproduced soluble protein due to the very high concentration of the protein in cellular extracts. In the case of CFA synthase, this is a valuable purification step giving concentration of the enzyme and a 3.5-fold purification with 70% activity retained.

Homogeneous CFA synthase can now be obtained in milligram quantities, allowing the novel topological and mechanistic properties of the enzyme to be studied. However, the purified enzyme remains extremely labile when not bound to phospholipid vesicles. This lability precludes determination of the native form of CFA synthase. Either the protein must remain bound to lipid vesicles, which invalidates any hydrodynamic measurements, or be released from vesicles, in which case an enzyme of compromised activity is studied. Radiation inactivation seems the most direct means to obtain the native molecular weight.

The amino acid sequence of CFA synthase shows no significant similarity to any protein of the Genbank, EMBL, or Swiss Protein data bases by the search alogarithim of Pearson and Lipman (1988). However, by use of the Motif program of Smith et al., (1990) we found small segments of sequence similarity between CFA synthase and a number of S-AdoMet-utilizing enzymes. Three distinct motifs were found among the 40 sequences examined (Figure 8). We

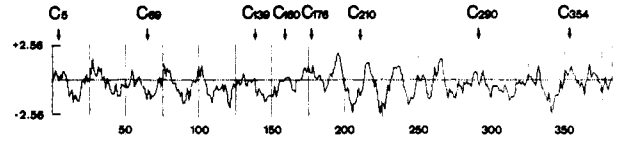

FIGURE 9: Hydrophobicity profile of the deduced amino acid sequence of CFA synthase. The hydrophobicity values are plotted against the position by method of Kyte and Doolittle (1982). Hydrophobic values are positive. The positions of the cysteine residues in the sequence are indicated.

found two sequences present in CFA synthase and in over half of the sequences available. These sequences, GxGG and DxxxGxG, which overlap in the region of CFA synthase between residues 171 and 182 (Figure 8), were found in 23 and 24 of the 41 sequences, respectively. Another sequence proposed to play a role in S-AdoMet binding, VL($^{E}/_{D}$)xGxGxG (Ingrosso et al., 1989; Haydock et al., 1991) is also found in this segment of CFA synthase and in a degenerate form (DxGxGxG) in several other proteins but was not present in many other S-AdoMet-utilizing enzymes. Thus, the VLDIGCGWGG sequence of CFA synthase (residues 171–182) contains three motifs found in other S-AdoMet utilizing proteins, DxxxGxG, GxGG, and VLDxGxGxG. Given that S-AdoMet binding is the only known property in common between CFA synthase and the other enzymes, the residue 171–182 segment of CFA synthase seems very likely to be involved in binding this substrate. It should be noted that CFA synthase lacks the phenylalanine residue of the S-AdoMet binding motif, FxGxG, proposed by both Lauster (1989) and Smith et al. (1990). However, this motif has been found only in methyltransferases that act on nucleic acids (Haydock et al., 1991). Moreover, cross-linking experiments in which S-AdoMet or its azido derivative have been used to identify S-AdoMet binding sites of DNA methyltransferases (Som & Friedman, 1991; Reich & Everett, 1990) have not yet detected binding to the regions of these proteins that contain this motif.

The *E. coli* CFA synthase seems to be a protein of moderate hydrophobicity, consistent with its isolation from the cellular cytosol. The protein lacks any of the long hydrophobic regions such as those seen in membrane proteins (Figure 9). The sequence, therefore gives no clues to the mechanism whereby the enzyme gains access to the acyl chain double bonds of phospholipid vesicles. By analogy to recent crystallographic structures of phospholipases and lipases (Brozowski et al., 1991; Scott et al., 1990; Winkler et al., 1991) and the prediction of Desnuelle et al. (1960), it seems likely that the active site of CFA synthase is located within the enzyme structure, rather than being exposed on the enzyme surface where the hydrophobic nature of the site could cause the enzyme to be poorly soluble and aggregate. We thus propose a model in which a conformational change that occurs upon lipid binding would stabilize the enzyme structure and expose the active site to the acyl chain double bond. The great increase in stability of the enzyme upon binding phospholipid vesicles is consistent with a major conformational change. However, CFA synthase must differ from the lipases in that it must penetrate much deeper into the lipid bilayer than do the lipase enzymes. Moreover, the enzyme seems to be able to "measure" the position of the double bond on the acyl chain (Marinari et al., 1974) and binds only to phospholipids containing either UFA or CFA chains. CFA synthase fails to bind to vesicles made of phospholipids containing only saturated or branched-chain fatty acids, and such phospholipids neither inhibit enzyme action nor protect the enzyme from loss of activity (Taylor & Cronan, 1979).

A first clue to the mechanism of CFA synthase action is our demonstration that a sulfhydryl group seems to be essential for activity. Some preliminary evidence for an essential sulfhydryl group was obtained previously by inhibition with other sulfhydryl reagents (Taylor, 1977), but the present data are more definitive. The essential sulfhydryl group was protected by addition of UFA- or CFA-containing phospholipids, suggesting that this group is located close to the phospholipid binding site. Another possibility is that phospholipid binding may change the conformation of the enzyme such that the sulfhydryl reagents no longer have access to the reactive cysteine. The increased rate of inactivation seen when the chain length of the inhibitor is increased indicates that the sulfhydryl group is located in a hydrophobic pocket or cleft sufficiently large to accommodate a C7 acyl chain. Eight cysteine residues are found in the amino acid sequence of CFA synthase (Figure 3). Two residues, C176 and C354, are located within hydrophobic segments of the primary sequence as predicted by the hydrophobicity profile plotted by the method of Kyte and Doolittle (1982) (Figure 9). C176 lies within the putative S-AdoMet binding site whereas C354 is near the C-terminus of CFA synthase. If the hydrophobic cleft is formed by a distinct segment of primary sequence, both of these cysteine residues seem to be reasonable candidates for providing an essential sulfhydryl group. However, if the putative S-AdoMet binding site is correct, C176 seems an unlikely candidate, since S-AdoMet failed to protect CFA synthase against inactivation by *N*-hexylmaleimide (Table III). C354 seems a stronger candidate since deletion of the last 50 residues of CFA synthase results in loss of all enzyme activity (data not shown). The importance of these residues will be addressed by in vitro site-directed mutagenesis.

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