Escherichia coli Cyclopropane Fatty Acid Synthase: Is a Bound Bicarbonate Ion the Active-Site Base?†

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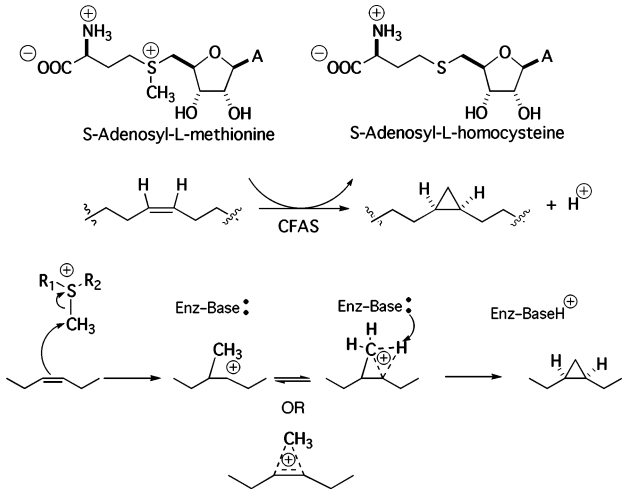
ABSTRACT: Cyclopropane synthases catalyze the cyclopropanation of unsaturated fatty acid using *S*-adenosyl-L-methionine as the methylene donor. The crystal structure of three cyclopropane synthases from *Mycobacterium tuberculosis* showed a bicarbonate ion bound in the active site that was proposed to act as a general base in the reaction mechanism [Huang, C., Smith, V., Glickman, M. S., Jacobs, W. R., and Sacchettini, J. C. (2002) *J. Biol. Chem. 277*, 11559−11569]. Because the in vitro activity of *M. tuberculosis* cyclopropane synthases has not yet been reported and because the ligands of the bicarbonate ion are all strictly conserved in cyclopropane synthases, we used the closely related *Escherichia coli* cyclopropane fatty acid synthase for this study. The putative ligands that share a hydrogen bond with the bicarbonate through their side chains were mutated. H266A, Y317F, E239A, and E239Q mutants were thus constructed and purified, and their catalytic efficiencies were 5.3, 0.7, 0.2, and <0.02%, respectively. C139 that is bound to the bicarbonate by its NH amide had already been mutated to serine in a previous work, and this mutant retains 31% of the activity of the wild-type enzyme. Kinetic analyses and binding studies using spectrofluorimetry showed that these mutations affected the catalytic constant rather than the binding of the substrates. While addition of free bicarbonate had almost no effect on the wild-type enzyme activity, all mutants, with the exception of E239A and E239Q, were rescued by the addition of free bicarbonate. The catalytic efficiencies of the rescued mutants were 85, 16, and 14% for C139S, H266A, and Y317F, respectively. This effect was specific to bicarbonate. The kinetic parameters of the rescued mutants were determined, and it is shown that the rescuing effect is due to an increase in k_{cat}. These data are interpreted by assuming that the *E. coli* cyclopropane fatty acid synthase specifically binds a bicarbonate ion that is involved in catalysis, as proposed for the *M. tuberculosis* enzymes, and that mutation of the bicarbonate ligands decreases the affinity for that ion. However, because the E239Q mutation could not be rescued, we propose that E239 forms a catalytic dyad with the bicarbonate to perform the proton abstraction necessary in the chemical pathway to the cyclopropane ring.

Cyclopropane synthases catalyze the cyclopropanation of unsaturated lipids in bacteria (*1*−*4*), plants (*5*, *6*), and parasites (*7*). In *Escherichia coli*, the enzyme known as cyclopropane fatty acid synthase (CFAS)[1] cyclopropanates the double bond of unsaturated phospholipids (Scheme 1). This chemical modification is thought to be involved in the long-term survival of nongrowing cells and is often associated with environmental stresses (*2*). In *Mycobacterium tuberculosis*, three enzymes are involved in cyclopropanations of mycolic acids, at different positions on the alkyl chain. In this case, the modification has recently been associated with virulence and persistence of the pathogen (*8*, *9*), making the *M. tuberculosis* cyclopropane synthases

Scheme 1: Reaction Catalyzed by Cyclopropane Synthases and Proposed Reaction Mechanism[a]



[a] The postulated intermediate is a stabilized carbocation or a protonated cyclopropane that is deprotonated by an active-site base.

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[1] Abbreviations: BSA, bovine serum albumin; CFAS, cyclopropane fatty acid synthase; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl *β*-D-thiogalactopyranoside; *S*-AdoMet, *S*-adenosyl-L-methionine; *S*-AdoHcy, *S*-adenosyl-L-homocysteine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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interesting therapeutic targets. While the three-dimensional structure of the *M. tuberculosis* cyclopropane synthases has been determined (*10*), their in vitro catalytic activity has not yet been fully characterized despite interesting attempts (*11*), probably because the structure of the actual lipid substrate is not known. In this context, the *E. coli* enzyme remains a good working model for both mechanistic (*12*) and inhibition studies (*13*). Indeed, the primary sequences of the cyclopropane synthases known so far exhibit high degree of identity, suggesting a conserved reaction mechanism (*2*, *3*).

Two reaction mechanisms that differ in the type of intermediate involved have been proposed for the enzymatic cyclopropanation: a carbocation mechanism (*2*, *3*, *14*) (Scheme 1) and a mechanism involving a sulfur ylide (*15*, *16*).

Recent experimental data obtained in different laboratories (*12*, *17*−*21*) have favored the carbocation mechanism which was originally proposed by Lederer (*14*): the π-electrons of the (*Z*)-double bond attack the methyl group of *S*-adenosyl-L-methionine (*S*-AdoMet) to give the carbocation intermediate that could be in rapid equilibrium with a protonated cyclopropane. A proton is then abstracted by an active-site base to yield the *cis*-cyclopropane ring (Scheme 1). Nevertheless, many aspects of this enzymatic reaction remain unclear. For example, it is not known how the cyclopropane synthases bring together *S*-AdoMet, the soluble substrate, and the highly hydrophobic lipid chain. In particular, in the case of the *E. coli* CFAS, the phospholipids are organized in such a way that the double bond is buried in the membrane bilayer. There are also some mechanistic details that are not known such as the exact structure of the intermediate and how the enzyme stabilizes such a species, as well as the identity of the active-site base that abstracts the proton.

The crystal structure of the *M. tuberculosis* enzymes reported by Sacchettini and colleagues (*10*) complexed with quaternary alkylammoniums, thought to mimic the intermediate, gave some clues regarding these issues: a tyrosine residue was found to be close enough to the positively charged nitrogen atom to be involved in a π-cation interaction with the carbocation intermediate, and an unexpected bicarbonate ion, found near the methyl group of the intermediate mimic, seemed to be almost ideally located for general base catalysis. Because bicarbonate is not commonly found in enzyme active sites, further experimental data are needed to support its assigned role in the cyclopropane synthases reaction mechanism.

We report here mutagenetic and chemical rescue experiments that confirm an important role for bicarbonate in *E. coli* CFAS catalysis. In addition, we propose that residue E239, one of the bicarbonate ligands, could form a catalytic dyad with this ion.

EXPERIMENTAL PROCEDURES

General. *E. coli* BL21(DE3) was obtained from Promega (Madison, WI). Synthetic oligonucleotides were products of Proligo (Paris, France) and were used without any further purification. Restriction enzymes and molecular biology kits were obtained either from Promega or from Roche (Meylan, France). Chemicals were purchased from Sigma-Aldrich (Saint Quentin, France) and were of the highest available purity. *S*-[*methyl*-^{14}C]Adenosyl-L-methionine (60 mCi/mmol) and *S*-[*methyl*-^{3}H]adenosyl-L-methionine (15 Ci/mmol) were from New England Nuclear (Boston, MA). Culture medium components were purchased from Difco Laboratories (Detroit, MI). Chromatographic equipment (GradiFrac) and column phases were from Amersham Biosciences (Orsay, France). UV−visible spectra were obtained on an Uvikon-930 Kontron spectrophotometer (Munich, Germany) or a Lambda-40 Perkin-Elmer (Norwalk, CT) apparatus. Scintillation counting was conducted on a 1214 Rackbeta LKB Wallac radioactivity counter (Perkin-Elmer). Sonication was performed on a VibraCell sonicator from Bioblock (Illkirch, France). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out on a Bio-Rad (Hercules, CA) Protean II system, using the conditions described by the manufacturer, and DNA electrophoresis on a Mupid apparatus (Eurogentec, Seraing, Belgium), in 40 mM Tris-acetate buffer (pH 7.5) and 1 mM EDTA. Centrifugations were run on a Sorval RF5plus centrifuge (DuPont, Kendro, Courtaboeuf, France). Fluorescence spectra were acquired on a fluorescence spectrometer from Photon Technology Internanional (Lawrenceville, NJ) with a xenon arc set at 58 W with slits set as follows: 4 nm at the entrance, 4 nm at the excitation monochromator, 4 nm at the emission monochromator, and 10 nm at the photomultiplier. Fluorescence data were acquired and processed using Felix software (Photon Technology International).

Site-Directed Mutagenesis. The mutated *cfa*E239A, *cfa*E239Q, *cfa*H266A, and *cfa*Y317F genes [numbering corresponds to the natural *E. coli* CFAS sequence, which is without counting the N-terminal six-histidine tag that has been engineered (*12*)] were constructed using the Quick Change site-directed mutagenesis kit from Stratagene (La Jolla, CA). The following sets of mutated primers (mutations are underlined) were used: E239A, 5′-CTGTGGGGATGTTCGCGCACGTCGGACCG-3′ and 5′-CGGTCCGACGTGCGCGAACATCCCCACAG-3′; E239Q, 5′-CTGTGGGGATGTTCCAGCACGTCGGACCG-3′ and 5′-CGGTCCGACGTGCTGGAACATCCCCACAG-3′; H266A, 5′-GGCATATTCCTGCTCGCTACTATCGGTTCG-3′ and 5′-CGAACCGATAGTAGCGAGCAGGAATATGCC-3′; and Y317F, 5′-CGGTGCTGATTTCGATACTACGTTGATGGC-3′ and 5′-GCCATCAACGTAGTATCGAAATCAGCACCG-3′. *cfa*C139S gene construction has already been described (*12*). Plasmid pET24H6cfa, containing the *E. coli* wild-type *cfa* gene, with an additionnal six-histidine tag sequence enginered at the 5′ end, was used as the template (*12*). Transformants were selected, and the plasmids were extracted, purified (Wizzard Plus miniprep kit from Promega), and sequenced [Eurogentec or MilleGen (Labège, France)] to ensure the presence of the desired mutation. Competent *E. coli* BL21(DE3) cells were then transformed by each mutated plasmid for protein production.

Expression and Purification of Wild-Type and Mutant CFAS. The six-histidine-tagged mutant proteins were obtained in pure form as described for the six-histidine-tagged wild-type enzyme (*12*). In the case of mutant H266A, the transformant was grown at 37 °C (up to an absorbance A_{600} of 0.7), and the culture was then transferred to a water bath at 20 °C prior to the addition of IPTG (100 μM final concentration) and shaken for an additional 36 h at 20 °C, to minimize the formation of inclusion bodies.

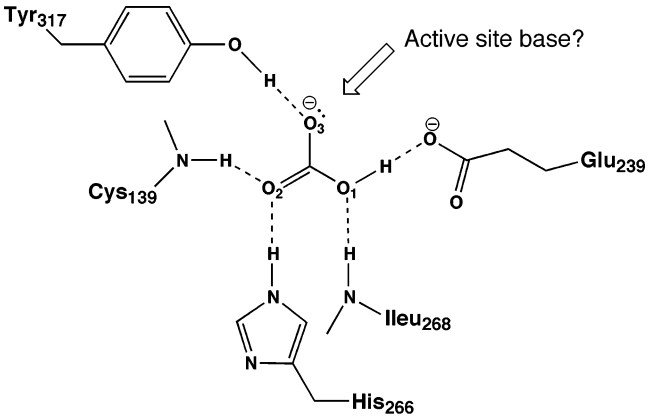
Protein Assay. Protein concentrations were determined using the colorimetric assay described by Bradford (*22*) and supplied by Bio-Rad, using bovine serum albumin (BSA) as a standard.

CFAS Activity Assay. The activity of wild-type and mutant CFAS was assayed as previously described (*12*). Briefly, the assay consisted of 1 mg/mL *E. coli* total phospholipids, 0.5 mg/mL BSA, 0.75 mM *S*-AdoMet, either ^{14}C-labeled or ^{3}H-labeled, and from 2 to 50 μg of CFAS, depending on the specific activity of the protein that was being tested, in a final volume of 100 μL. However, instead of using 20 mM potassium phosphate buffer (pH 7.4) as originally reported (*12*), we used here 100 mM HEPES buffer (pH 8.0). This modification was important to keep the pH constant during the experiments run in the presence of bicarbonate. The reaction was initiated by addition of the enzyme, the mixture incubated at 37 °C for 15 min, and the reaction stopped by addition of 1 mL of 10% (w/v) trichloroacetic acid. The radioactivity associated with the phospholipid fraction was then determined as described previously (*12*). Specific activities were carefully determined by measuring the slope of a linear plot of the activity, measured using the conditions described above, against the concentration of the enzyme that was being tested. One unit of CFAS is defined as the amount of enzyme that transforms 1 μmol of substrate per minute. These specific activity values were used to estimate the k_{cat} of the slowest mutants, that is, Y317F, E239A, and E239Q CFAS. Kinetic parameters of individual enzymes were determined by measuring the activity (as described above) at various concentrations of *S*-AdoMet. Data were analyzed using a nonlinear regression analysis supported by Kaleidagraph (Synergy Software, Reading, PA), to fit to Michaelis−Menten kinetics. Saturating concentrations of phospholipids were determined by measuring the CFAS activity at various phospholipid concentrations (0 to 2 mg/mL). When required, buffers and stock solutions were repeatedly degassed in vacuo and flushed with ultrapure argon (U-Ar, from Messer-France, Paris, France).

Effect of Bicarbonate and Other Ions on CFAS Activity. The effects of bicarbonate and other salts on wild-type and mutant CFAS were determined by measuring the catalytic activity as described above, at different salt concentrations in the assay mixture. Stock solutions of the different salts (0.1−1 M) were prepared in water, and the pH was adjusted to 8.0 ± 0.1 by adding concentrated HCl or NaOH. Na_2SO_4 and $NaNO_3$ stock solutions were directly prepared in 100 mM HEPES buffer (pH 8.0). The kinetic parameters of wild-type, C139S, H266A, and Y317F CFAS were determined in the presence of sodium bicarbonate at pH 8.0, at the following concentrations: 10 mM for wild-type CFAS, 50 mM for C139S and H266A CFAS, and 100 mM for Y317F CFAS.

Binding Studies Using Spectrofluorimetry. Dissociation constants of *S*-AdoHcy for wild-type and mutant CFAS were determined by following the decrease in the intrinsic fluorescence of the protein upon binding of the ligand. All measurements were taken in a 10 mm × 40 mm quartz cuvette at 15 °C. The excitation wavelength was set at 290 nm, and the emission fluorescence was recorded at 330 nm. Small volume aliquots of the titrant in stock solution [30 mM *S*-AdoHcy in 50% (v/v) EtOH/H_2O] were added to the protein sample [0.02 mg/mL in 20 mM potassium phosphate (pH 7.4)] under continuous stirring. After correction for dilution, the data points [relative fluorescence intensities ($F_0 - F$)/F_0 against *S*-AdoHcy concentrations] were fitted to a hyperbolic saturation curve using nonlinear regression analysis supported by Kaleidagraph.

Scheme 2: Representation of the Bicarbonate Binding Site in Cyclopropane Synthases[a]



[a] Adapted from the crystal structure of *M. tuberculosis* enzymes (*10*). The bound bicarbonate was proposed, on structural grounds, to be the active-site base. Numberings are those of *E. coli* CFAS.

RESULTS

Production and Purification of the Mutated CFAS. An unexpected electron density observed in the active site of crystallized *M. tuberculosis* cyclopropane synthases was assigned to a bound bicarbonate ion, hydrogen bonded to five active-site residues (*10*). These residues, C139, I268, E239, H266, and Y317 (*E. coli* CFAS numbering), are strictly conserved in all cyclopropane synthases (*2*, *3*), suggesting an important role for the bicarbonate ion (Scheme 2). Furthermore, in the crystal structure, O3 of the bicarbonate ion is close (3.1 Å) to the methyl group of a bound lipid analogue that mimics the carbocation formed during the reaction. It thus led Sacchettini and colleagues (*10*) to postulate that the bicarbonate could be the active-site base.

We thus mutated these bicarbonate ion ligands to explore this hypothesis. Y317 was mutated to F317 to remove the phenolic function while keeping the aromatic ring. H266 was mutated to Ala, while E239 was mutated to Ala and to Gln. The two other ligands (NH amide) cannot be replaced since they are part of the polypeptide backbone. However, because we had in hand mutant C139S (*12*) we also studied that replacement in this report. All mutant genes were obtained by PCR amplification using two sets of mutated primers, and the Stratagene QuickChange technology. The desired mutations were all verified by DNA sequencing. The mutated proteins were expressed and purified as described for the wild-type enzyme (*12*), except for the H266A mutant protein which precipitated as inclusion bodies, under those conditions. We thus optimized the overexpression conditions and obtained the soluble protein under the following induction conditions: 100 μM IPTG, 20 °C, and incubation for 36 h. H266A CFAS was then successfuly purified as described for the wild-type enzyme.

Kinetic Characterization of the CFAS Mutants. Wild-type and mutant CFAS were assayed as we previously described (*12*), except that we used, in the work described here, 100

Table 1: Kinetic and Binding Parameters for Wild-Type and Mutant CFAS[a]

enzyme	K_d for *S*-AdoHcy (μM)	K_m for *S*-AdoMet (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	relative catalytic efficiency (%)
wild-type	51 ± 8	70 ± 2	2.52 ± 0.07	36.0	100
C139S	61 ± 11	75 ± 3	0.85 ± 0.06	11.3	31
H266A	220 ± 5	78 ± 20	0.15 ± 0.02	1.9	5.3
Y317F	30 ± 3	ND[b]	ND[b]	ND[b]	0.7[c]
E239A	66 ± 13	ND[b]	ND[b]	ND[b]	0.2[c]
E239Q	152 ± 8	ND[b]	ND[b]	ND[b]	<0.02[d]

[a] Dissociation constants were obtained from fluorescence experiments, and represent the average of three independent measurements. Kinetic parameters were derived from activity measurements and represent the average of three independent experiments (see Experimental Procedures for details). [b] Not determined. [c] Value estimated from the ratio of specific activities rather than from the ratio of specificity constants. [d] No activity could be measured for that mutant, using our radioactive assay. We thus can only give an upper limit estimated to be 0.02% of the wild-type enzyme activity.

mM HEPES buffer (pH 8.0) instead of 20 mM potassium phosphate buffer (pH 7.4) to simplify the comparison with the chemical rescue experiments in the presence of bicarbonate. Under these slightly different conditions, we measured for the wild-type enzyme a K_m of 70 ± 2 μM for *S*-AdoMet and a k_{cat} of 2.52 ± 0.07 min^{-1}, values in close agreement with those previously reported in phosphate buffer (pH 7.4) [$K_m = 90$ μM, $k_{cat} = 2.2$ min^{-1} (*12*)]. As the second substrate of CFAS is an insoluble phospholipid, this enzyme belongs to the interfacial enzyme family. Therefore, the K_m for the insoluble substrate cannot be derived from the conventional initial rate versus molar concentration plot, but rather by using an interfacial area per unit volume as the insoluble substrate concentration. This analysis has not yet been reported for CFAS, and we did not perform it, because it requires the use of pure phospholipids and calibrated vesicles. However, as reported by Cronan and co-workers (*23*), we observed a biphasic dependence of the velocity of the reaction over the phospholipid concentration, with a quasi-linear dependence up to 0.5 mg/mL phospholipids followed by saturation at higher concentrations. We thus used a saturating phospholipid concentration of 1 mg/mL throughout this study. Under these conditions, the kinetic parameters for the soluble substrate can be derived using the usual plots. Table 1 summarizes the kinetic parameters for individual mutants and for the wild-type enzyme. C139S and H266A mutants are relatively catalytically active, 30 and 5.2% of the wild-type specificity constant k_{cat}/K_m, respectively. While their K_m for *S*-AdoMet was only slightly affected, indicating a normal binding of the substrate, the mutation lowered the catalytic constant. Y317F and E239A are significantly impaired (0.8 and 0.2%, respectively), and E239Q mutant activity is at the background level of the assay used in this report (less than 0.02% of the activity of the wild-type enzyme). For these last three mutants, it was not possible to obtain precise kinetic constants, and thus, the catalytic efficiency of these mutants was calculated using the ratio of their specific activity over that of the wild-type enzyme. However, as shown below, we proved that these mutants are able to bind the product of the reaction, and therefore, it is reasonable to postulate that the mutation greatly affects the k_{cat}. We also checked that the mutations did not affect the binding to the phospholipids. As for the wild-type

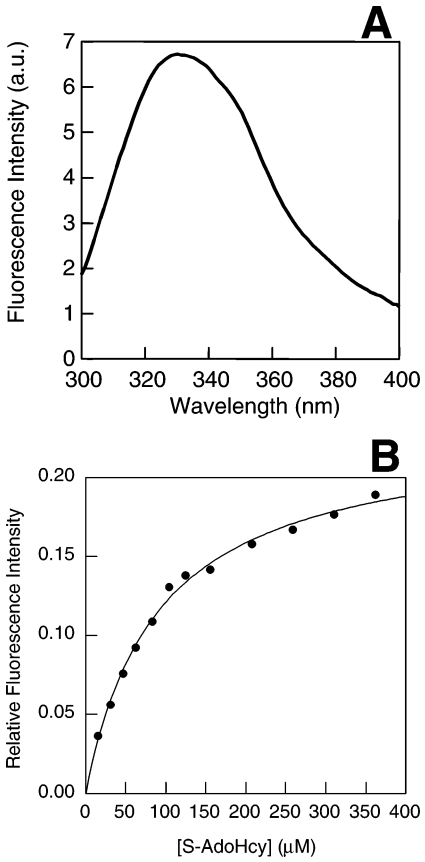


FIGURE 1: Determination of the dissociation constants for *S*-AdoHcy using fluorescence spectroscopy. (A) Emission fluorescence spectrum of wild-type CFAS recorded at 15 °C [20 μg/mL in 20 mM potassium phosphate buffer (pH 7.4), excitation at 290 nm]. (B) Quenching of the intrinsic fluorescence of E239A CFAS by *S*-AdoHcy. Increasing amounts of *S*-AdoHcy were added to a solution of E239A CFAS [20 μg/mL in 20 mM potassium phosphate buffer (pH 7.4)], and the fluorescence was measured after excitation at 290 nm and 15 °C. After correction for dilution, the relative fluorescence intensity $(F_0 - F)/F_0$ was plotted against the *S*-AdoHcy concentration, and the data points were fitted to a hyperbolic saturation curve.

enzyme, we observed for C139S and H266A CFAS a biphasic curve with saturation around 0.5 mg/mL when the velocity was plotted against the phospholipid concentration. It thus appears that mutating the bicarbonate ligands has a deleterious effect on the enzyme activity, confirming an important role for the bicarbonate ion.

Dissociation Constant Determination Using Fluorescence Spectroscopy. Because simple kinetic analysis did not allow the determination of K_m and k_{cat} parameters in the case of the slowest mutants (Y317F, E239A, and E239Q CFAS), we studied the binding of the soluble substrate and the soluble product using spectrofluorimetry, taking advantage of the presence of 11 tryptophans in the enzyme. Figure 1A shows the emission fluorescence spectrum of wild-type CFAS when excitation was set at 290 nm. This particular excitation wavelength was chosen to prevent a strong inner filter effect by absorption by the adenine-containing titrant. The emission spectrum shows a maximum at 330 nm that is typical of tryptophan fluorescence. We noted that above 25 °C, the fluorescence emission at 330 nm of wild-type CFAS decreased with time: 30% of the fluorescence emission is lost within 30 min, an observation probably related to the short half-life of CFAS in solution (*23*). We thus had to

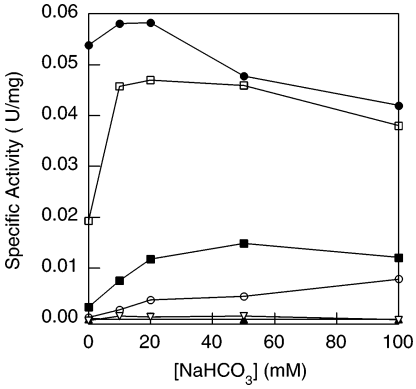


FIGURE 2: Effect of increasing concentrations of sodium bicarbonate on wild-type and mutant CFAS activity. The specific activity of the CFAS was measured as described in Experimental Procedures in the presence of increasing amounts of sodium bicarbonate: wild type (●), C139S (□), H266A (■), Y317F (○), E239A (▲), and E239Q (▽). Data points are connected by lines for clarity. Each point represents the average of duplicate experiments with a <5% dispersion. Error bars are not shown for clarity.

acquire the data at 15 °C, a temperature at which the fluorescence emission was stable in the time range of the experiment. When *S*-AdoMet was used as a titrant, we obtained only poor quality data. However, using *S*-AdoHcy, the product of the reaction, as the titrant, we obtained satisfactory results: a decrease in fluorescence intensity (20%) was observed upon binding at saturation. Figure 1B shows a typical binding experiment, where the relative fluorescence intensity is plotted against the titrant concentration. Fitting the data to a hyperbolic saturation curve gave an estimate of the dissociation constant. This experiment was repeated for all the mutants described in this work (Table 1). *S*-AdoHcy is a competitive inhibitor of the reaction with a K_I of 30 μM (*13*, *23*, *24*). The dissociation constant independently measured in this report ($K_D = 51$ μM for wild-type CFAS) is consistent with that value. Y317F and E239A CFAS bind *S*-AdoHcy as well as the wild-type enzyme does, suggesting a correct folding of the proteins with an unperturbed substrate binding site. H266A CFAS and E239Q CFAS exhibited a slightly lower affinity for *S*-AdoHcy. However, the values obtained here suggest that all these mutants bind the product and that their low activity is very likely due to an impaired catalytic step rather than a poor binding of the substrate.

Rescuing the Impaired Mutants with Bicarbonate. Because the crystal structure of *M. tuberculosis* cyclopropane synthases showed the presence of a bicarbonate ion in the active site (*10*), we tested the effect of increasing concentrations of free bicarbonate at a constant pH on the activity of the *E. coli* enzyme. As shown in Figure 2, the specific activity of wild-type CFAS is barely affected by the presence of bicarbonate at concentrations lower than 20 mM. At higher concentrations, there is a slight inhibitory effect (20% inhibition at 100 mM).

Attempts to prepare the wild-type apoenzyme, that is, the enzyme devoid of bicarbonate, were unsuccessful. Degassing the protein solution and buffers did not affect the activity of the wild-type enzyme. For C139S, H266A, and Y317F mutants, there is a clear activating effect with sodium bicarbonate. The concentration at which the effect is maximal depends on the individual mutant: 50 mM for C139S and H266A CFAS and 100 mM for Y317F CFAS. The activity of the E239A and E239Q mutants was not restored by the presence of sodium bicarbonate, which suggests a specific role for E239. We checked, on wild-type and mutant CFAS, that this activating effect was specific to bicarbonate by replacing $NaHCO_3$, in the assay mixture, with various salts, at different concentrations. Ammonium bicarbonate gave the same effect that sodium bicarbonate did, showing that the nature of the counterion is unimportant. Sodium acetate, ammonium formate, sodium nitrate, sodium sulfate, or sodium sulfite had no effect: the specific activities were not different (variation of <5%) in the presence or absence of the ions. Thus, the bicarbonate activating effect is specific and suggests a specific role for that ion in the catalyzed reaction.

Table 2: Kinetic Parameters of Wild-Type and Bicarbonate-Rescued Mutant CFAS[a]

enzyme	K_m for *S*-AdoMet (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	relative catalytic efficiency (%)[b]
wild-type	70 ± 2	2.77 ± 0.05	39.6	100
C139S	57 ± 8	1.92 ± 0.07	33.7	85
H266A	61 ± 7	0.58 ± 0.06	6.5	16
Y317F	60 ± 5	0.34 ± 0.02	5.6	14

[a] Kinetic parameters were derived from activity measurements (see Experimental Procedures for details) in the presence of 10 mM sodium bicarbonate for wild-type CFAS, 50 mM sodium bicarbonate for C139S and H266A CFAS, and 100 mM sodium bicarbonate for Y317F CFAS. Reported values represent the average of duplicate experiments. [b] Ratio of k_{cat}/K_m constants for bicarbonate-rescued mutants over the k_{cat}/K_m constant measured for the wild-type enzyme in the presence of 10 mM $NaHCO_3$.

The kinetic parameters of wild-type and mutant CFAS were determined in the presence of bicarbonate at the concentration that gave the highest rescuing effect, that is, 10 mM for wild-type CFAS, 50 mM for C139S and H266A CFAS, and 100 mM for Y317F CFAS (Table 2). It is clear from the data presented in Table 2 that the presence of sodium bicarbonate has only a very moderate effect on the K_m of *S*-AdoMet, but a large effect on the k_{cat}. Comparing data reported in Tables 1 and 2, one can see that the catalytic constants are enhanced by factors of 1.1, 2.2, and 3.9 for wild-type, C139S, and H266A CFAS, respectively. The catalytic efficiencies of the mutants are also greatly enhanced by factors of 2.7, 3.0, and 20 for C139S, H266A, and Y317F CFAS, respectively. Interestingly, mutants E239A and E239Q could not be rescued. The fact that the bicarbonate rescuing effect is mainly seen on the catalytic constants strongly suggests that this ion is involved in catalysis rather than in binding. Since Y317F is rather active in the presence of 100 mM sodium bicarbonate, we checked that, under these conditions, binding to phospholipids was not impaired. As for the wild-type enzyme and the other active mutants, we observed a biphasic curve when velocity was plotted against phospholipid concentration with a breaking point at 0.5 mg/mL, which suggests a normal binding to the insoluble substrate.

DISCUSSION

Cyclopropane synthases catalyze the transfer of a methylene group from *S*-AdoMet to an unactivated carbon−carbon double bond of a fatty acid (*1*−*3*). Several lines of

evidence obtained in different laboratories (*12*, *17*−*21*) support the carbocation mechanism for this unusual reaction, as schematically depicted in Scheme 1. While this reaction mechanism was rapidly proposed by Lederer (*14*), alternative mechanisms, namely, the addition of an ylide (*15*) or a carbenoid species (*16*) to the double bond, were suggested. In vivo feeding experiments favored the carbocation mechanism (*17*−*19*). However, in vitro experiments became possible only when purification of the enzyme proved to be practicable (*25*). Characterization of the pure *E. coli* enzyme showed that the enzyme is devoid of an organic or metallic cofactor (*20*, *21*, *12*), that no exchange of the methyl proton of *S*-AdoMet is observed (*12*), and that the reactivity of seleno- and telluro-AdoMet, as substrates, is best interpreted by invoking a methyl transfer rather than the formation of an ylide species (*21*). Furthermore, the three-dimensional structures of three putative cyclopropane synthases from *M. tuberculosis*, complexed with carbocation mimics, have been determined and gave firm support to the formation of a carbocation intermediate in the reaction (*10*). Unexpectedly, Sacchettini and co-workers (*10*) observed the presence of a bicarbonate ion in the active site of the three enzymes. They proposed, on structural grounds, that this bicarbonate could be the active-site base that abstracts a proton in the second step of the accepted reaction mechanism (Scheme 1). Unfortunately, the lipid substrate of the *M. tuberculosis* enzymes has not yet been characterized, thus preventing in vitro experiments. On the other hand, the structure of the *E. coli* enzyme has not yet been determined, but the high degree of sequence identity between the cyclopropane synthases allows simple speculation. For instance, one can reasonably state that the active-site structures are conserved as well as the reaction mechanism.

The bicarbonate ion observed in the *M. tuberculosis* enzymes active site is bound through hydrogen bonds to five residues, two of which are bound through their N−H polypeptide backbone amide (*10*) (Scheme 2). In this work, we have mutated the three side chain ligands, in the *E. coli* enzyme, to suppress the hydrogen bonds to the bicarbonate ion: H266A, Y317F, and E239A mutants were thus prepared. All these mutations led to slow mutants, the slowest being E239A. Interestingly, mutant E239Q, thereafter constructed, was even less active, even though the amide side chain is a hydrogen bond donor or acceptor. Binding experiments using spectrofluorimetry showed that all mutants are still able to bind *S*-AdoHcy, the product of the reaction. We also verified that the mutants were able to bind to the phospholipids just as the wild-type enzyme did. It is thus clear that the mutations primarily affect the catalytic machinery of these enzymes.

Most important is the fact that addition of free bicarbonate, and no other structurally related ion, to these mutants led to a large increase in specific activity, except for E239A and E239Q. Kinetic analysis showed that this specific rescuing effect is the consequence of an increase in the catalytic constants. This effect was also observed for the C139S mutant that we had previously prepared (*12*). This mutant is only slightly less active than the wild-type enzyme (the catalytic efficiency is 31%), but addition of 50 mM bicarbonate increases its catalytic constant by a factor of 2. This weak effect is not surprising since this mutation does not remove the N−H bond, but perhaps shifts it very slightly. It is also interesting to note that the less active the mutant is, the less important the bicarbonate rescuing effect is. The simplest interpretation of these data is that the *E. coli* CFAS contains a binding site for bicarbonate and that mutation of any of the bicarbonate ligands decreases the affinity for that ion. It was not possible to measure a binding constant for bicarbonate on the wild-type enzyme because the apoenzyme could not be prepared by degassing buffers and protein solutions. This suggests that the affinity for the bicarbonate ion, in the wild-type enzyme, should be very high. For the H266A mutant, it was possible to extract an apparent K_m of 25 mM, from the data reported in Figure 1. For the other mutants, the curve could not be fitted to a simple saturation process. More detailed information about the binding of bicarbonate to the *E. coli* enzyme must await the resolution of the X-ray structure of wild-type and mutant CFAS.

The fact that E239A and E239Q could not be rescued by bicarbonate suggests a specific role for E239. On one hand, a simple interpretation would be that the mutation decreases the affinity for the bicarbonate in such a way that this ion at 100 mM is not sufficient to observe a small activity. On the other hand, it is difficult to imagine that if E239 serves only as a ligand, its replacement with a glutamine would not yield a partially active mutant. The crystal structure of the *M. tuberculosis* enzymes showed that E239 is too far from the methyl group of the quaternary ammonium salt used as the carbocation analogue to be the base that is needed in the second step of the reaction mechanism (*10*). We thus propose that this glutamic residue forms a catalytic dyad together with the bicarbonate, to assist the latter in the deprotonation step.

Bicarbonate is not commonly found in the enzyme active site, except when it is the actual substrate as in carboxylases or in carbonic anhydrase. A striking example is the dizinc leucine aminopeptidase isolated from bovine lens, for which a bicarbonate was proposed to be the active-site base that removes a proton from the water molecule bound to the zinc ions (*26*, *27*). The bicarbonate binding site, in that enzyme, as revealed by X-ray crystallography, is quite different from that described for the cyclopropane synthases. In leucine aminopeptidase the bicarbonate is bound to an arginine side chain, to two polypeptide backbone amides, and to one polypeptide backbone carbonyl. For leucine aminopeptidase, a partially bicarbonate depleted enzyme could be prepared by degassing, and the authors could measure an apparent K_m of 0.21 mM for bicarbonate (*27*). The differences in binding site, in leucine aminopeptidase and cyclopropane synthases, could explain the different affinities for bicarbonate, assuming a very tight binding for bicarbonate in the latter.

The bicarbonate-stimulated adenylyl cyclase is a member of another class of enzymes that shows activation by bicarbonate (*28*). However, in that case, the bicarbonate ion seems to be a regulator or a sensor rather than a cofactor that directly participates in catalysis, and the apparent binding constant for bicarbonate is 25 mM (*29*), 2 orders of magnitude higher than the one measured for leucine aminopeptidase. Nevertheless, bicarbonate is an important biomolecule and will certainly be found, in the future, to play important roles in other enzymes.

While this article was in review, a paper by Booker and colleagues was published (*30*). They provided evidence that

the activity of *E. coli* CFAS depends on the presence of bicarbonate. They elegantly succeeded in preparing the *E. coli* CFAS apoenzyme by consuming the bicarbonate present in the enzyme solution using phosphoenolpyruvate carboxylase and malate dehydrogenase at pH 8.0. The apparent binding constant for bicarbonate was measured and found to be 49 μM. In our study, we were unable to prepare the apoenzyme by simply degassing the solution probably because at pH 8.0 the release of bicarbonate is too slow and has to be accelerated by enzymatic consumption. In vivo, the wild-type enzyme is probably saturated by intracellular bicarbonate, whereas the mutants described herein are produced as apoenzymes because they need higher concentrations to be saturated. Booker and colleagues also briefly reported the preparation of mutants (H266A, Y317F, E239A, and E239D) but did not report their characterizations. Overall, our results and theirs are complementary and unambiguously show that bicarbonate is bound to the *E. coli* CFAS and that it participates in catalysis. The exact role of the bicarbonate (general base catalysis or intermediate stabilization by electrostatic catalysis) cannot be definitely defined at the moment. However, we think, in accordance with the proposition by Sacchettini (*10*), that the bicarbonate acts as a base in the active site. Indeed, even though the postulated protonated cyclopropane intermediate has a low pK_a, the proton will be captured by a base since it is very acidic. The closest base is the bicarbonate, and it is a good candidate for general base catalysis. The assistance by E269 in the deprotonation step is a proposition we make on the basis of some mutagenesis experiments, and we will confirm this by determining the three-dimensional structure of the *E. coli* CFAS.

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