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Research paper

An active site mutant of *Escherichia coli* cyclopropane fatty acid synthase forms new non-natural fatty acids providing insights on the mechanism of the enzymatic reaction

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ABSTRACT

We have produced and purified an active site mutant of the *Escherichia coli* cyclopropane fatty acid synthase (CFAS) by replacing the strictly conserved G236 within cyclopropane synthases, by a glutamate residue, which corresponds to E146 of the homologous mycolic acid methyltransferase, Hma, producing hydroxymethyl mycolic acids. The G236E CFAS mutant had less than 1% of the *in vitro* activity of the wild type enzyme. We expressed the G236E CFAS mutant in an *E. coli* (DE3) strain in which the chromosomal *cfa* gene had been deleted. After extraction of phospholipids and conversion into the corresponding fatty acid methyl esters (FAMEs), we observed the formation of cyclopropanated FAMEs suggesting that the mutant retained some of the normal activity *in vivo*. However, we also observed the formation of new C17 methyl-branched unsaturated FAMEs whose structures were determined using GC/MS and NMR analyses. The double bond was located at different positions 8, 9 or 10, and the methyl group at position 10 or 9. Thus, this new FAMEs are likely arising from a 16:1 acyl chain of a phospholipid that had been transformed by the G236E CFAS mutant *in vivo*. The reaction catalyzed by this G236E CFAS mutant thus starts by the methylation of the unsaturated acyl chain at position 10 or 9 yielding a carbocation at position 9 or 10 respectively. It follows then two competing steps, a normal cyclopropanation or hydride shift/elimination events giving different combinations of alkenes. This study not only provides further evidence that cyclopropane synthases (CSs) form a carbocationic intermediate but also opens the way to CSs engineering for the synthesis of non-natural fatty acids.

1. Introduction

Bacterial cyclopropane synthases (CS) constitute an interesting family of enzymes catalyzing the transfer of a methylene group from *S*-adenosyl-L-methionine (AdoMet) to an unactivated double bond of a fatty acid chain leading to the formation of a cyclopropane ring (Fig. 1) [1]. Two principal classes of bacterial CS have attracted particular attention: *Escherichia coli* cyclopropane fatty acid synthase (CFAS) that uses unsaturated phospholipids as substrates, and *Mycobacterium tuberculosis* cyclopropane mycolic acid synthases (CMASs) which perform the introduction of cyclopropane rings at proximal and distal positions of unsaturated mycolic acids [2–5]. The physiological role of lipid cyclopropanation in *E. coli* remains unclear, but several hypotheses have been proposed, including modification of membrane fluidity, which would be

Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; CS, cyclopropane synthase; CFAS, cyclopropane fatty acid synthase; CMAS, cyclopropane mycolic acid synthase; DMDS, dimethyl disulfide; DMSO, dimethylsulfoxide; EI, electron impact; FAME, fatty acid methyl ester; GC, gas chromatography; GC/MS, gas chromatography coupled to mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside.

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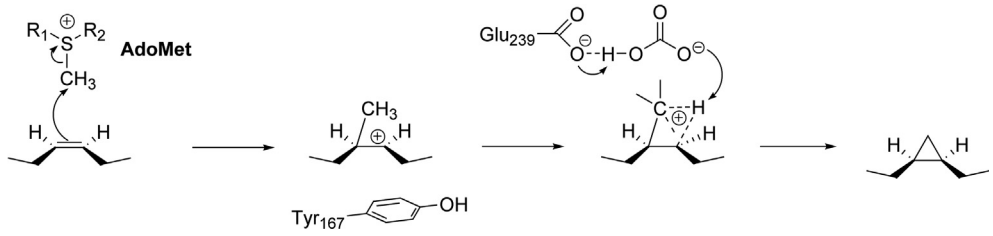

Fig. 1. Reaction catalyzed by cyclopropane synthases, and the proposed reaction mechanism. The amino acid numbering corresponds to that of the *E. coli* CFAS. The stereochemistry of the process has been elucidated and the configuration of the product is 9*R*,10*S* [38].

beneficial for cell survival, or higher resistance toward acid shock, or other environmental stresses [1]. In *M. tuberculosis*, the CMASs seem to be implicated in pathogenicity thus making them attractive targets for new antituberculosis drugs [4,6–8]. CMASs and CFAS share up to 33% sequence identities suggesting a common reaction mechanism. While the three dimensional structures of three different *M. tuberculosis* CMASs (PcaA, CmaA1 and CmaA2) are available [9], their *in vitro* activity has not yet been reported. This is very likely due to the fact that the exact structure of their substrates is not yet known. It has however been proposed that these methyltransferases act on a fatty acid chain linked to an acyl-carrier protein [10–13]. In contrast, the *E. coli* CFAS enzyme has been extensively studied *in vitro*, but no structural data is yet available. The accepted overall reaction mechanism of the *E. coli* CFAS involves the formation of a carbocationic intermediate, by methylation of the double bond, that is stabilized by pi–cation interactions with Y167, followed by a deprotonation step very likely catalyzed by a bicarbonate ion bound in the active site (Fig. 1) [14,15]. This reaction mechanism is thus a very good working model for all bacterial cyclopropane synthases and the *E. coli* CFAS constitutes the only available *in vitro* model system to date for enzymatic and inhibition studies on this class of enzymes [16,17]. In addition to CMASs, other AdoMet-dependant methyltransferases are implicated in the modification of mycolic acids. Among them, Hma (also named Mma4 or CmaA), is necessary for the biosynthesis of keto- and methoxy-mycolic acids [18]. It apparently produces hydroxymethyl(mero)mycolates [19], which would be transformed into methoxy(mero)mycolates by the *O*-methyltransferase Mma3 or into keto(mero)mycolates by oxidation [3,10,20]. The role of keto- and methoxy-mycolic acids is crucial since they are necessary for *M. tuberculosis* virulence in mouse [4,6,21]. The crystallographic structure of Hma, obtained recently, has been compared to that of CMASs [22]. As expected, for enzymes belonging to the same family, most of the active site residues or residues in contact to AdoMet are conserved. However and quite interestingly, the bicarbonate ion found in the active site of CMASs is replaced, in Hma, by the side chain carboxylate of E146. This carboxylate is actually placed at exactly the same position as the carbonate, on the superimposed structures. It was thus tentatively proposed that E146 may function as a general base to activate a water molecule in the reaction mechanism of Hma (Fig. 2) [22]. As originally proposed by Lederer [23], it is tempting to state that the catalysis by CS and mycolic acid methyltransferases, would follow the same route to the carbocation intermediate and then differ in the second step leading to the product. Thus, CMASs and Hma, that probably use the same substrate, would catalyze the formation of the same carbocation intermediate. The final product would then depend on the nature of the residues at the active site: (*i*) the presence of a general base, the carbonate, positioned to abstract a proton from the methyl group leading to cyclization in the case of CMAS and hence CFAS; or (*ii*) a base activating a water molecule for a nucleophilic attack [18,19,21] leading to the hydroxylated and methylated product in the case of Hma (Fig. 2). Because E146 of Hma is replaced by a conserved glycine in all *M. tuberculosis* CMASs and in *E. coli* CFAS (Fig. S1), we thus decided to mutate the G236 of *E. coli* CFAS into a glutamate residue to test if this mutation would change the course of the reaction.

2. Materials and methods

2.1. Materials

E. coli K12 JW1653*cfa*$^-$ was obtained from the Keio collection (Institute for Advanced Biosciences, Keio University, Tsuruoka City, Yamagata, Japan) [24]. Plasmid pCP20 encoding the Flp recombinase was obtained from Dr. Lionello Bossi (Centre de Génétique Moléculaire, Gif sur Yvette, France). *E. coli* BL21(DE3)/pET24(+)H$_6$-*cfa* strain overproducing the *E. coli* CFAS, was obtained as previously described [25]. Chemicals were purchased from Sigma–Aldrich (Saint-Quentin, France) and were of the highest purity available.

2.2. Site-directed mutagenesis

The G236E mutation in the *cfa* gene was obtained by using the site-directed mutagenesis kit from Stratagene. The template used was the plasmid pET24-H6*cfa* and the following sets of mutated primers (mutations are underlined) were used for the PCR amplification: 5′-GAT CGT ATT GTT TCT GTG GAG ATG TTC GAG CAC GTC-3′ and 5′-GAC GTG CTC GAA CAT CTC CAC AGC AAC AAT ACG ATC-3′. A transformant was selected, and the plasmid was extracted, purified and sequenced to ensure the presence of the desired mutation. Competent *E. coli* BL21(DE3) cells were then transformed with the mutated plasmid, pET24-H6*cfa*G236E, for protein production.

2.3. Expression and purification of wild-type and mutant CFAS

The six-histidine-tagged mutant G236E CFAS was obtained in pure form as described for the six-histidine-tagged wild-type enzyme [25].

2.4. Engineering of the E. coli K12*cfa*$^-$(DE3) strain

E. coli K12*cfa*$^-$ was transformed with the plasmid pCP20 with a temperature-sensitive replication origin for temporal production of the yeast Flp recombinase. Transformants were selected in the presence of ampicillin at 30 °C and culturing of transformants at 37 °C led to the loss of pCP20. Colonies of *E. coli* were cultured in the presence of ampicillin and kanamycin at 37 °C and those showing sensitivity to both antibiotics were selected. The *E. coli* K12 *cfa*$^-$(DE3) strain was constructed using a λ DE3 lysogenization kit (Novagen) for site-specific integration of λ DE3 prophage into *E. coli.*

2.5. CFAS activity assay

The activities of wild-type and mutant CFAS were assayed as previously described [25]. Briefly, the assay consisted of 1 mg/mL