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Short communication

Insight into the reaction mechanism of the *Escherichia coli* cyclopropane fatty acid synthase: Isotope exchange and kinetic isotope effects

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ABSTRACT

Cyclopropanation of unsaturated lipids is an intriguing enzymatic reaction and a potential therapeutic target in *Mycobacterium tuberculosis*. Cyclopropane fatty acid synthase from *Escherichia coli* is the only *in vitro* model available to date for mechanistic and inhibition studies. While the overall reaction mechanism of this enzymatic process is now well accepted, some mechanistic issues are still debated. Using homogeneous *E. coli* enzyme we have shown that, contrary to previous report based on *in vivo* experiments, there is no exchange of the cylopropane methylene protons with the solvent during catalysis, as probed by ultra high resolution mass spectrometry. Using [*methyl*-^{14}C]-labeled and [*methyl*-$^{3}H_3$]-*S*-adenosyl-L-methionine we have measured a significant intermolecular primary tritium kinetic isotope effect ($^{T}V/K_{app} = 1.8 \pm 0.1$) consistent with a partially rate determining deprotonation step. We conclude that both chemical steps of this enzymatic cyclopropanation, the methyl addition onto the double bond and the deprotonation step, are rate determining, a common situation in efficient enzymes.

1. Introduction

Bacterial cyclopropane synthases (CS) constitute an interesting family of enzymes that catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to an unactivated double bond of a lipid chain to form of a cyclopropane ring (Fig. 1) [1]. This reaction is interesting because it is a difficult chemical transformation and because *Mycobacterium tuberculosis* CSs, which cyclopropanate unsaturated mycolic acids [2], are attractive targets for new antituberculous drugs [3,4]. The *Escherichia coli* cyclopropane fatty acid synthase (CFAS) that acts on unsaturated phospholipids is the best characterized enzyme of this class and we have shown that it is a good model for bacterial CSs. By screening for inhibitors of the *E. coli* CFAS we identified antituberculous compounds that target *M. tuberculosis* CSs and related methyl transferases [5–8].

The overall reaction mechanism of the *E. coli* CFAS, now accepted, involves the formation, by methylation of the double bond, of a carbocationic intermediate that is stabilized by pi-cation interactions, followed by a deprotonation step very likely catalyzed by a bicarbonate ion bound in the active site, assisted by a conserved glutamate residue (Fig. 1) [9–17]. Because all bacterial CSs share significant sequence identity the *E. coli* CFAS reaction mechanism represents a very good working model for CSs. Furthermore, this enzyme is the only *in vitro* system available to date for enzymatic and inhibition studies on CSs. However, some mechanistic issues remain unclear or unsolved. Buist et al. [18–20] measured no intermolecular kinetic isotope effect ($k_H/k_D = 1.01 \pm 0.04$) using [*methyl*-$^{2}H_3$]-methionine and *in vivo* feeding experiments with *Lactobacillus plantarum*. Booker et al. [13,14], using pure *E. coli* CFAS and *in vitro* noncompetitive conditions with [*methyl*-$^{2}H_3$]-AdoMet, measured an inverse secondary kinetic isotope effect ($^{D}V = 0.87 \pm 0.08$). These data were interpreted as the consequence of a slow methyl transfer (step 1 in the reaction mechanism) and a rapid second step. The *in vivo* experiments, described by Buist et al. were complicated by the fact that a substantial exchange (33%) of the cyclopropane methylene hydrogens, with the solvent protons, occurred during the catalyzed reaction. The extent of this exchange varied with the pH of the culture medium, suggesting a complex process. Because these isotope exchange and kinetic isotope effect experiments were preformed *in vivo*, with the possibility of unexpected artifact, or *in vitro* but using noncompetitive conditions that are known to be not very accurate and error-prone (over 5% [21–23]) we have decided to reexamine these mechanistic issues.

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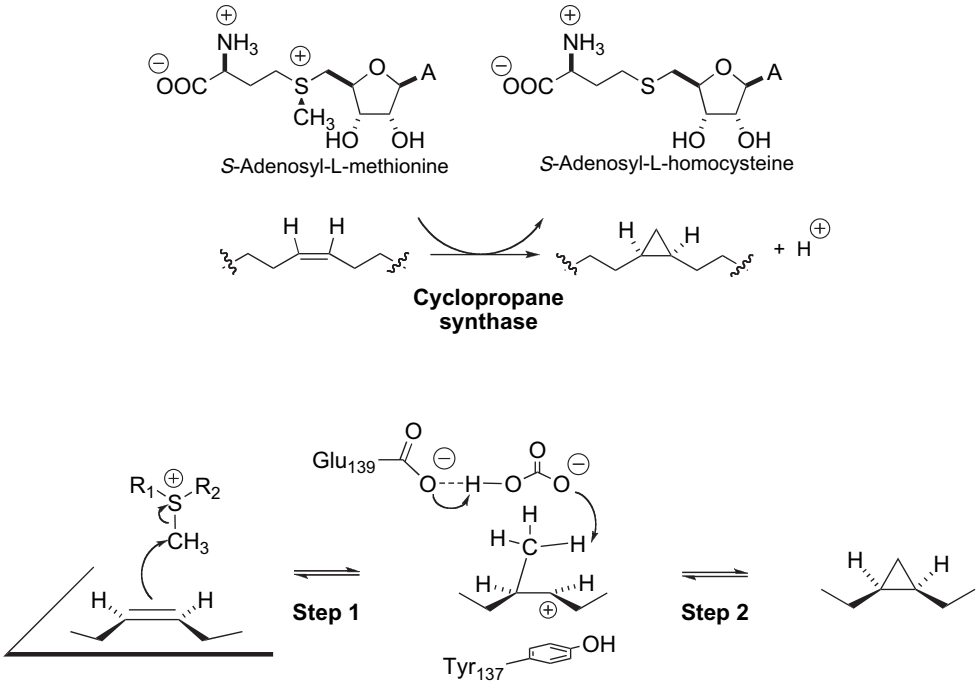

Fig. 1. The reaction catalyzed by cyclopropane synthases and the reaction mechanism proposed for the *E. coli* cyclopropane fatty acid synthase. The depicted amino acid side chains refer to the *E. coli* enzyme.

2. Materials and methods

2.1. E. coli CFAS preparation and assay

The recombinant histidine tagged CFAS from *E. coli*, the adenosylhomocysteine nucleosidase and the unsaturated phospholipids from *E. coli* K12 were purified and assayed as already described [6].

2.2. Deuterium exchange experiments and quantification of the exchange by ultra high resolution mass spectrometry

A typical experiment consisted of: 750 μM AdoMet, 1.0 mg mL^{-1} phospholipids, 0.25 mg mL^{-1} bovine serum albumin, 0.1 μM adenosylhomocysteine nucleosidase, 0.3 mg mL^{-1} CFAS, 10 mM potassium phosphate buffer, pH 7.4 in a final volume of 1.0 mL. A pre-mixture without the enzymes, was lyophilized and resuspended in D_2O, twice, to exchange the protons. The enzymes were then added to the mixture, so that the volume of H_2O over that of D_2O did not exceed 3%. Two control experiments, one in H_2O and one in D_2O without CFAS, were run at the same time. The three samples were incubated for 30 min at 37 °C and quenched by adding trichloroacetic acid to a final concentration of 0.1 M. The phospholipids were extracted with 800 μL of *n*-hexane, three times, and the n-hexane layers were pooled and incubated at room temperature with 200 μL of 2 M NaOH in methanol for 10 min. The fatty acid methyl esters (FAMEs) were then concentrated under a stream of N_2, and identified and quantified by gas chromatography coupled to mass spectrometry (GC-MS) and gas chromatography (GC) [6]. The extent of exchange was determined by high resolution positive electrospray mass spectrometry (p-ESI) using a hybrid linear ion trap LTQ-Orbitrap (Thermo Fisher Scientific, Les Ulis, France). FAMEs samples (0.5 μg) were introduced into the ionization chamber under atmospheric pressure through a stainless steel capillary *via* the LTQ-Orbitrap syringe pump at the rate of 5 μL min^{-1}. The electrospray voltage was set to 5 kV, the capillary voltage and the tube lens offset were set to 40 V and 80 V, respectively. The maximum injection time was 200 ms. The sheath and auxiliary gas flows (both nitrogen) were optimized at 15 and 5 (arbitrary units) and the drying gas temperature was set to 220 °C. The mass resolving power was set at the maximum value for the Orbitrap analyzer, that is, 10^5 full width at half maximum height at *m/z* 400). Each mass spectrum was the average of 80 scans (2 min accumulation).

2.3. Kinetic isotope effect experiments

Kinetic isotope effects were determined using the radioactive assay for CFAS as already described [12], except that a mixture of *S*-[*methyl*-^{3}H]-AdoMet (New England Nuclear, 85 Ci $mmol^{-1}$ or 10 Ci $mmol^{-1}$) and *S*-[*methyl*-^{14}C]-AdoMet (New England Nuclear, 60 Ci $mmol^{-1}$) was used. The actual specific radioactivity of the AdoMet used was, after isotopic dilution, 1.5 mCi $mmol^{-1}$ for *S*-[*methyl*-^{3}H]-AdoMet and 5 mCi $mmol^{-1}$ for *S*-[*methyl*-^{14}C]-AdoMet. The ^{14}C and ^{3}H labeling (dpm) of the phospholipids produced at different time points of the reaction were determined by using a dual counting mode (1214 Rackbeta LKB Wallac) and by correcting the data as described [24].

3. Results and discussion

3.1. Deuterium exchange experiment

The observed exchange reported by Buist et al. [18–20] could be explained by a reversible formation/opening of the cyclopropane ring in the active site (step 2 in Fig. 1) with a concomitant rapid exchange of the protonated active-site base with the solvent protons. Rapid rotation of the methyl group, in the active site, during this exchange, would result in an incorporation of the solvent protons on the methylene position of the cyclopropane of the product. To monitor any such exchange during the *E. coli* CFAS catalyzed reaction, we have performed the cyclopropanation in D_2O (containing 3% residual H_2O) and searched for any deuterium