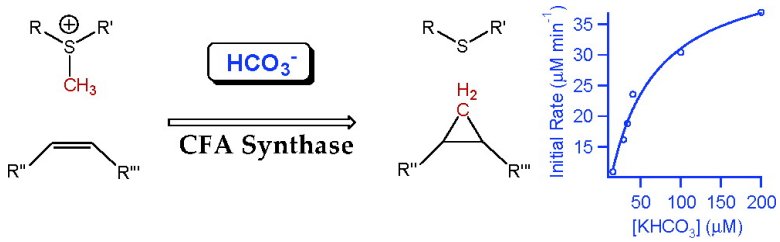
Communication

The Activity of *Escherichia coli* Cyclopropane Fatty Acid Synthase Depends on the Presence of Bicarbonate

David F. Iwig, Akira Uchida, Jeffrey A. Stromberg, and Squire J. Booker

J. Am. Chem. Soc., **2005**, 127 (33), 11612-11613• DOI: 10.1021/ja053899z • Publication Date (Web): 30 July 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 07/30/2005

The Activity of *Escherichia coli* Cyclopropane Fatty Acid Synthase Depends on the Presence of Bicarbonate

David F. Iwig, Akira Uchida, Jeffrey A. Stromberg, and Squire J. Booker*

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received June 13, 2005; E-mail: sjb14@psu.edu

Cyclopropane fatty acid (CFA) and cyclopropane mycolic acid (CMA) synthases catalyze the formation of cyclopropane rings on isolated and unactivated olefinic bonds within various fatty acids. The methylene carbon of the cyclopropane ring is derived from the activated methyl group of (*S*)-adenosyl-L-methionine (AdoMet), affording (*S*)-adenosyl-L-homocysteine and a proton as the two remaining products (Scheme 1).[1,2] Recently, use of kinetic isotope effects in combination with elemental effects have shown that the reaction takes place via a rate-limiting methyl transfer from AdoMet to the isolated olefin, presumably affording a secondary carbocation intermediate adjacent to a tertiary carbon center. Removal of a proton from this intermediate allows for subsequent cyclization. Alternatively, the carbocation intermediate might rearrange to a protonated cyclopropane ring from which the proton is lost (Scheme 1).[3,4] In vivo[5] and other in vitro[6,7] studies are also consistent with a carbocation mechanism; however, the manner by which the enzyme stabilizes the secondary carbocation intermediate has not been elucidated.

Cyclopropane rings also appear as one of several functional groups occurring on mycolic acids from several species of Mycobacterium. Interestingly, they are found almost exclusively in virulent species of Mycobacterium as opposed to saprophytic species.[8] Moreover, strains of *M. tuberculosis* possessing null mutations in *pcaA*, the gene that encodes the enzyme that cyclopropanates the proximal double bond in α-mycolic acids, cannot persist inside infected mice, suggesting that this CMA synthase might be a fruitful target for the design of antituberculosis agents.[9]

Crystal structures of three CMA synthases (CmaA1, CmaA2, and PcaA) have recently been reported.[10] Surprisingly, the active sites of each of the enzymes contained electron density that was attributed to a bicarbonate ion from its planar trigonal structure and hydrogen-bonding pattern. The bicarbonate ion was liganded via side chains contributed by His167, Glu140, and Tyr232 (CmaA1 numbering system)—each of which is conserved in the *E. coli* CFA synthase as well as other CMA synthases—and by the backbone amides of Cys35 and Ile169. The absolute conservation of amino acids that form the hydrogen-bonding network to the bicarbonate ion would suggest that the presence of the small molecule is not an artifact of the crystallization process. The ultimate indicator of the relevance of the bicarbonate ion, however, would be the dependence of CMA synthase activity on its presence. Unfortunately, a functional in vitro assay for CMA synthases has not been reported, primarily because the exact identity of the substrate for the reaction has not been established.[10] By contrast, substrates for CFA synthases are commercially available. *E. coli* CFA synthase shares ∼30−35% sequence identity with CmaA1, CmaA2, and PcaA, suggesting that their mechanisms of catalysis are conserved.[10] In this study, we show that the CFA synthase reaction is dependent upon bicarbonate, displaying Michaelis−Menton behavior with a K_M value of 49 ± 5.9 μM. Moreover, borate, a molecule that mimics the planar trigonal structure of bicarbonate, is a competitive inhibitor of the CFA synthase reaction, displaying a K_I value of 2.02 ± 0.290 mM. Last, we show that substitutions of amino acids in CFA synthase that correspond to those that ligand the bicarbonate ion in the CMA synthase structures result in greatly diminished activity.

Scheme 1. Cyclopropane Fatty Acid Synthase Reaction

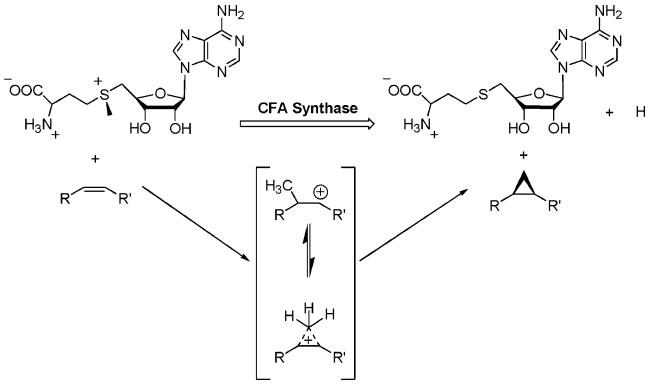


Table 1. Effect of Bicarbonate on the CFA Synthase Reaction[a]

purification buffer /assay buffer	K_M (AdoMet) (μM)	V_{max} $[E_T]^{-1}$ (min^{-1})
HEPES/HEPES	88.7 ± 18.9	6.24 ± 0.53
HEPES/$KHCO_3$	71.6 ± 8.6	21.0 ± 0.9
$KHCO_3$/HEPES	70.5 ± 12.9	23.0 ± 1.5
$KHCO_3$/$KHCO_3$	99.7 ± 13.4	19.1 ± 1.1

[a] Assays were carried out as described in Supporting Information. Error is reported as ± standard deviation.

The *E. coli cfa* gene was expressed from plasmid pATG100 as described previously,[3] and the his-tagged protein was isolated in the presence and absence of potassium bicarbonate. Both isolations were carried out simultaneously (50 mM potassium bicarbonate buffer, pH 7.0, replaced the normal 50 mM HEPES isolation buffer, pH 7.0, for the purification in the presence of bicarbonate), and both buffers were prepared simultaneously from the same source of deionized water. As indicated in Table 1, CFA synthase isolated in HEPES buffer and subsequently assayed in HEPES buffer (70 mM, pH 7.0) displayed V_{max} $[E_T]^{-1}$ and K_M values with respect to AdoMet that were similar to those previously reported.[3] When enzyme isolated in HEPES buffer was assayed in potassium bicarbonate buffer (70 mM, pH 7.0), there was greater than a 3-fold enhancement in V_{max} $[E_T]^{-1}$ with no significant change in K_M. When CFA synthase was isolated in potassium bicarbonate buffer but assayed in 70 mM HEPES buffer, pH 7.0, the activity of the enzyme was similar to that observed when isolated in HEPES buffer and assayed in potassium bicarbonate buffer (Table 1); however, the concentration of bicarbonate in the assay mixture attributed to addition of enzyme was ∼2 mM. As expected, CFA synthase isolated and assayed in potassium bicarbonate buffer displayed the

10.1021/ja053899z CCC: $30.25

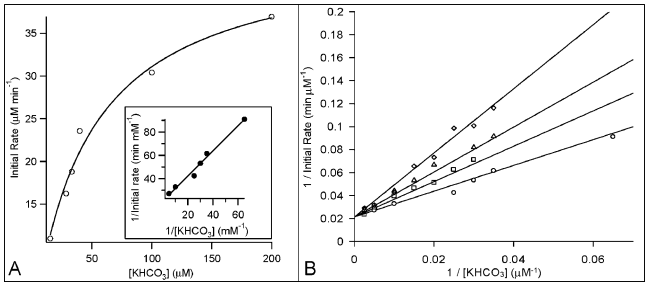


Figure 1. (A) The effect of $[HCO_3^-]$ on the CFA synthase reaction. After removing $[HCO_3^-]$ from the reaction mixture, the scrubbing system was quenched by addition of EDTA (7.5 mM). A given amount of $[HCO_3^-]$ was added, and the CFA synthase reaction was initiated by addition of AdoMet. (Inset) Lineweaver−Burk representation of the data. (B) Inhibition of the CFA synthase reaction by borate. Bicarbonate was removed from solution as described above, and then added back at varying concentrations in the presence of 0 mM (circles), 0.75 mM (squares), 1.5 mM (triangles), and 3 mM (diamonds) borate.

Table 2. Effect of Individual Components of the Bicarbonate Scrubbing System on CFA Synthase Activity[a]

component omitted from assay	relative activity
all components omitted	1.00
PEP	1.03
$MgCl_2$	0.962
PEPCase	1.04
none	<0.03

[a] Assays were carried out as described in Supporting Information.

more than 3-fold higher activity. Again, K_M values for AdoMet remained relatively constant under all conditions.

To better assess and quantify the effect of bicarbonate on the CFA synthase reaction, phosphoenolpyruvate carboxylase (PEPCase) and malate dehydrogenase (MDH) were used to remove or "scrub" the reaction mixture of bicarbonate to allow for reintroduction of known amounts before initiating assays. PEPCase catalyzes the carboxylation of phosphoenolpyruvate, affording oxaloacetate and inorganic phosphate. The oxaloacetate is subsequently reduced to malate concomitant with oxidation of NADH to NAD in a reaction catalyzed by MDH, allowing for removal of essentially all bicarbonate from solution. Because PEPCase is dependent on Mg^{2+}, the scrubbing system can be "turned off" by adding EDTA (7.5 mM) to the reaction, which has no effect on CFA synthase activity.[3] Assays were carried out at pH 8.0, which is the pH optimum for PEPCase. At pH values less than 7.5, the activity of PEPCase decreases significantly, while pH-rate profiles conducted by us (data not shown) and others[6] indicate that there is no significant difference in V_{max} for the CFA synthase reaction in the pH regime of 7.0−8.0.

The effect of the bicarbonate scrubbing system on CFA synthase activity is shown in Table 2. The activity of CFA synthase, determined in the absence of all components of the scrubbing system, was assigned a value of 1.00 (100%). When all components of the scrubbing system were present, CFA synthase activity was less than 3% of the activity obtained when all components of the scrubbing system were omitted. When PEP, $MgCl_2$, or PEPCase were omitted from the reaction mixture, conditions under which the scrubbing system is not operative, the relative activities were 1.03, 0.962, and 1.04, respectively.

The ability to remove bicarbonate from solution allowed for its reintroduction to assays in defined amounts. The dependence of the CFA synthase reaction on bicarbonate followed Michaelis−Menton kinetics (Figure 1A), displaying K_M and V_{max} $[E_T]^{-1}$ values of 49.3 ± 5.9 μM and 20.0 ± 1.0 min^{-1}, respectively. Various small molecules that mimic the planar trigonal structure of bicarbonate (urea, nitrate, formate, borate, and trithiocarbonate) were tested as inhibitors of the CFA synthase reaction. Only borate significantly inhibited the reaction, which was competitive in nature, yielding a K_I value of 2.02 ± 0.29 mM (Figure 1B).

CFA synthase variants H266A, Y317F, E239A, and E239D were constructed[11] and then purified as described for the wild-type protein. These amino acids are conserved among the CFA and CMA synthases and correspond to those that ligand the bicarbonate ion in the CMA synthase crystal structures.[10] Each of these variants was assayed in the presence of 3 mM $KHCO_3$, considerably above the K_M value for bicarbonate that we established for the wild-type enzyme. The H266A variant had 2.1% the activity of the wild-type protein, while the Y317F, E239A, and E239D variants had 0.45, 0.57, and 0.96% of the activity, consistent with their presumed roles as ligands to the bicarbonate ion. Studies are currently underway to characterize these variants in more detail.

Our finding that the CFA synthase reaction is dependent upon bicarbonate raises the issue of its role, if any, in catalysis. Certainly, it may simply play a structural role in the enzyme; however, its proximity to the functionalized portion of the substrate analogues that were co-crystallized with the CMA synthases suggests that its role is more intricate. Indeed, it has been suggested that it may act as the base that removes the proton from the carbocation intermediate (Scheme 1), a role that has precedence in the biochemical literature.[12] In accord with this premise, published pH-rate (V_{max}) profiles of the CFA synthase reaction yielded a pK_a value (6.8) consistent with the ionization of a bicarbonate ion. However, in vivo[5] and in vitro[3] studies indicate that methyl transfer, and not proton abstraction, is rate-limiting in the reaction. Therefore, the bicarbonate ion may simply act electrostatically to stabilize the developing carbocation intermediate. Efforts are currently in progress to sort out the details of this exciting and important reaction.

Acknowledgment. This research was supported by a Presidential Early Career Award in Science and Engineering to S.J.B. sponsored by the National Science Foundation (MCB-0133826), as well as an REU supplement to J.A.S.

Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Grogan, D. W.; Cronan, J. E., Jr. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 429−441.
(2) Law, J. H. *Acc. Chem. Res.* **1971**, *4*, 199−203.
(3) Iwig, D. F.; Grippe, A. T.; McIntyre, T. A.; Booker, S. J. *Biochemistry* **2004**, *43*, 13510−13524.
(4) Iwig, D. F.; Booker, S. J. *Biochemistry* **2004**, *43*, 13496−13509.
(5) Buist, P. H.; MacLean, D. B. *Can. J. Chem.* **1982**, *60*, 371−378.
(6) Courtois, F.; Guérard, C.; Thomas, X.; Ploux, O. *Eur. J. Biochem.* **2004**, *271*, 4769−4778.
(7) Molitor, E. J.; Paschal, B. M.; Liu, H.-W. *ChemBioChem* **2003**, *4*, 1352−1356.
(8) Chatterjee, D. *Curr. Opin. Chem. Biol.* **1997**, *1*, 579−588.
(9) Glickman, M. S.; Cox, J. S.; Jacobs, W. R. *Mol. Cell* **2000**, *5*, 717−727.
(10) Huang, C. C.; Smith, C. V.; Glickman, M. S.; Jacobs, W. R.; Sacchettini, J. C. *J. Biol. Chem.* **2002**, *277*, 11559−11569.
(11) Variants were constructed using the Stratagene QuikChange Mutagenesis Kit. Details will be described in a later publication.
(12) Strater, N.; Sun, L.; Kantrowitz, E. R.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11151−11155.

JA053899Z