Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2879–2881

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Cryptoregiochemical Analysis of an Unusual Bacterial Desaturation

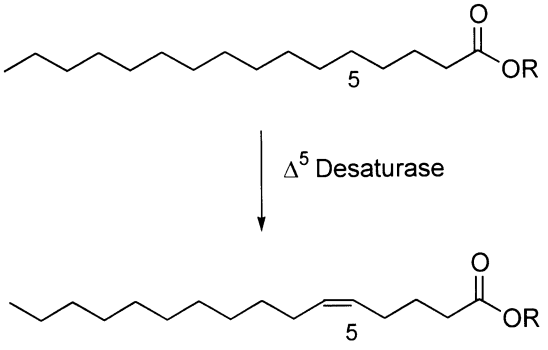
Laëtitia Fauconnot and Peter H. Buist*

Ottawa-Carleton Chemistry Institute, Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

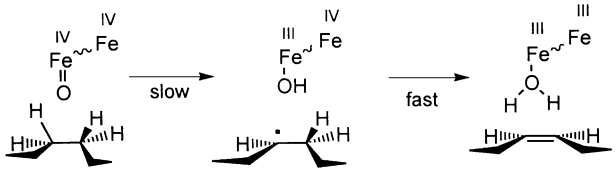
Received 6 June 2001; accepted 14 August 2001

Abstract—The cryptoregiochemistry of the cold-induced Δ^5 desaturation of long chain fatty acids, as it occurs in *Bacillus subtilis* ATCC 23857, has been examined by measuring the individual primary deuterium kinetic isotope effects associated with the C–H bond cleavage at C-5 and C-6. The results point to C-5 as the site of initial oxidation in Δ^5 desaturation. © 2001 Elsevier Science Ltd. All rights reserved.

Modification of long-chain fatty acyl derivatives constitutes one of the important cellular responses to various external stimuli. An interesting example of this phenomenon is the *cis*-Δ^5 desaturation of cell membrane phospholipids in various species of *Bacillus*.[1a,b] This process is induced when the temperature of bacterial cultures are lowered from 37 to 20 °C; the resultant increase in the proportion of *cis*-unsaturated fatty acyl chains in the cell membrane allows the appropriate fluidity of the lipid bilayer to be maintained.[2] What is unique about the *Bacillus* desaturase system is the unusual position of the newly introduced *cis*-double bond—namely, between carbons 5 and 6 rather than at the more common 9,10-position. Thus cellular palmitate is converted to *cis*-5-hexadecenoate upon cold shock treatment.[3] Sequence analysis of the gene encoding the Δ^5 desaturase[4] in *Bacillus subtilis* reveals that this protein occupies its own niche in the large family of O_2-dependent, membrane-bound, diiron-containing desaturases.[5] As part of a research program dedicated to correlating desaturase regioselectivity with protein structure, we were interested in pinpointing which substrate methylene group is attacked first by the Δ^5 desaturase. We have developed[6] a versatile KIE method for 'cryptoregiochemical' analyses of this type based on the assumption that the energetically difficult, first C–H activation step in desaturation is kinetically more important and hence more sensitive to deuterium substitution than the second C–H bond cleavage (Scheme 1).[7] Corroborating evidence for the location of the oxidant relative to substrate has been obtained in a number of cases.[7–12] Herein, we describe the application of our approach to the study of the Δ^5 desaturase in *B. subtilis*.



Our method of measuring the primary deuterium KIE for fatty acid desaturase-mediated oxidations involves the mass spectral analysis of products derived from a direct competition experiment between the parent substrate and a regiospecifically dideuterated analogue.[6]

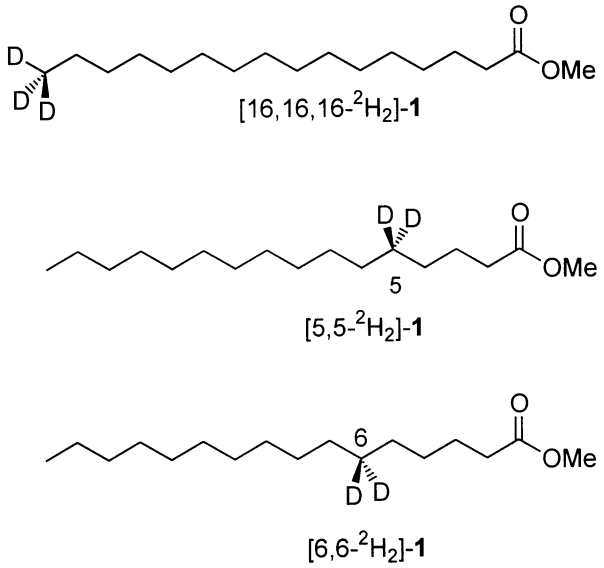


Scheme 1.

*Corresponding author. Tel.: +1-613-520-2600x3643; fax: +1-613-520-3749 or 3830; e-mail: peter_buist@carleton.ca

PII: S0960-894X(01)00579-0

This technique is particularly useful when the desaturase in question is unstable outside of its natural cellular environment and/or is unusually difficult to reconstitute[13]—as is the case for nearly all membrane-bound desaturases studied to date. In order to determine whether our methodology was feasible in the case of the Δ^5 desaturase system, a trial experiment using a palmitoyl substrate, labelled with deuterium in a position remote to the site of oxidation, was carried out. Thus [16,16,16-$^2H_3$]-methyl palmitate[14] [16,16,16-$^2H_3$]-**1** (40 mg/L) was incubated with cultures of *B. subtilis* ATCC 23857, which were grown in a RB medium[15] at 37 °C to exponential phase and then at 20 °C for another 48 h. GC/MS analysis[16] of the fatty acids isolated from the centrifuged cells via a standard hydrolysis/methylation sequence[9] showed that exogenously supplied labelled palmitate was cleanly converted to the corresponding Δ^5 desaturated material in approximately 10% yield—a level of conversion which is suitable for the purposes of a KIE experiment run in the competitive mode.[17] In addition, no products of ω-hydroxylation, which might compromise the use of [16,16,16-$^2H_3$]-**1** as a reference standard, were detected.



The intermolecular primary deuterium KIE on each C–H cleavage step of Δ^5 desaturation was then determined by repeating the above experiment using equimolar mixtures of each dideuterated substrate with the reference substrate: [5,5-$^2H_2$]-**1**/[16,16,16-$^2H_3$]-**1** and [6,6-$^2H_2$]-**1**/[16,16,16-$^2H_3$]-**1** (40 mg/L). Use of [16,16,16-$^2H_3$]-**1** as the reference substrate in these competition experiments eliminates interference by endogenous (d_0)-palmitate in the mass spectral analysis of the products. Product kinetic isotope effects (k_H/k_D) for *cis*-5-hexadecenoate formation were calculated using the ratio [% d_3 (product)/% d_1 (product)]/[% d_3 (substrate)/% d_2 (substrate)] (Table 1). This analysis indicates the presence of a significant primary deuterium isotope effect[18] (3.9 ± 0.4) for the C–H bond cleavage at C-5 while the C_6–H bond breaking step was shown to be insensitive to deuterium substitution (KIE = 1.17 ± 0.02). According to our mechanism (Scheme 1), these results suggest that the site of initial oxidation for Δ^5 desaturation is at C-5.

Table 1. Intermolecular isotopic discrimination in Δ^5 desaturation of [5,5-$^2H_2$]-palmitate and [6,6-$^2H_2$]-palmitate by *B. subtilis*

Isotopic ratio[a]				
Substrates		Products		KIE[b]
$16d_3$:$5d_2$	$16d_3$:$6d_2$	$16d_3$:$5d_1$	$16d_3$:$6d_1$	
0.90 ± 0.01		3.5 ± 0.4		3.9 ± 0.4
	1.00 ± 0.01		1.17 ± 0.02	1.17 ± 0.02

[a]The isotopic ratio of each species is given as an average value based on three to four GC/MS runs.
[b]The average KIE (two incubations) ± standard deviation.

The results presented in this paper represent the first mechanistic study of a unique bacterial desaturase and set the stage for the development of Δ^5-specific probes/inhibitors. In this context, it would be interesting to compare the cryptoregiochemistry of the *Bacillus* enzyme with that of other Δ^5 desaturases such as the catalyst involved in arachidonic acid biosynthesis.[19]

Acknowledgements

We wish to thank the NSERC for financial support of this work. The award of a PDF scholarship by the region Brittany, France, to L.F. is gratefully acknowledged. We are also indebted to Dr. Clem Kazakoff (University of Ottawa) who performed the MS measurements. All deuterated materials were generously provided to us by Dr. P. S. Covello, PBI, Saskatoon, SASK, Canada.

References and Notes

1. (a) Fulco, A. J. *Biochim. Biophys. Acta* **1967**, *144*, 701. (b) Grau, R.; de Mendoza, D. *Mol. Microbiol.* **1993**, *8*, 535.
2. Barton, P. G.; Gunstone, F. D. *J. Biol. Chem.* **1975**, *250*, 4470.
3. Fulco, A. J.; Levy, R.; Bloch, K. *J. Biol. Chem.* **1964**, *239*, 998.
4. Aguilar, P. S.; Cronan, J. E.; Mendoza, D. *J. Bacteriol.* **1998**, *180*, 2194.
5. Shanklin, J.; Whittle, E.; Fox, B. G. *Biochemistry* **1994**, *33*, 12787.
6. Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1996**, *118*, 6295.
7. A more complete discussion of the mechanistic alternatives for desaturation and its relationship to hydroxylation[20] is found in: Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1998**, *120*, 871.
8. Broun, P.; Shanklin, J.; Whittle, E.; Somerville, C. *Science* **1998**, *282*, 1315.
9. Fauconnot, L.; Buist, P. H. *J. Org. Chem.* **2001**, *66*, 1210.
10. Buist, P. H.; Marecak, D. M. *J. Am. Chem. Soc.* **1992**, *114*, 5073.
11. Rettie, A. E.; Boberg, M.; Rettenmeier, A. W.; Baillie, T. A. *J. Biol. Chem.* **1988**, *263*, 13733.
12. Savile, C. K.; Fabrias, G.; Buist, P. H. *J. Am. Chem. Soc.* **2001**, *123*, 4382.
13. Ozols, J. *Mol. Biol. Cell* **1998**, *9*, 3445.
14. All of the required deuterated substrates were originally synthesized by the late Pat Tulloch, PBI, Saskatoon, Canada: Tulloch, A. P. *Prog. Lipid Res.* **1983**, *22*, 235.

15. RB medium (1 L) contains: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g.
16. The GC/MS analyses were carried out essentially as reported previously.[9] The GC retention times for methyl palmitate and methyl *cis*-5-hexdecenoate were 14:17 min and 13:54 min, respectively. Isotopic ratios were determined using the following ions: m/z 270, M^+ (methyl palmitate); m/z 194, $(CH_3(CH_2)_9CH{=}CH{-}CH{=}CH_2)^+$ (methyl *cis*-5 hexadecenoate).
17. Melander, L.; Saunders, W. H. In *Reaction Rates of Isotopic Molecules*; R. E. Krieger: FL, 1987; p 91.
18. It should be noted that a secondary deuterium KIE may also be operating but this effect is expected to be small ($<10\%$ of primary KIE).[21]
19. Watts, J. L.; Browse, J. *Arch. Biochem. Biophys.* **1999**, *362*, 175.
20. Austin, R. H.; Chang, H-K.; Zylstra, G. J.; Groves, J. T. *J. Am. Chem. Soc.* **2000**, *122*, 11747.
21. Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 2171.