Stereochemical Analyses of the *Streptomyces hygroscopicus* var. *ascomyceticus* Type-II Dehydroquinate Dehydratase and Evidence for a Role of the Enzyme in the Biosynthesis of the Shikimate-Derived Moiety of Ascomycin

Galina Florova,† Yuko M. Lindsay,† Maria S. Brown,‡ Hamish A. I. McArthur,‡ Claudio D. Denoya,‡ and Kevin A. Reynolds*,†

Institute for Structural Biology and Drug Discovery and Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, Virginia 23219, and Bioprocess Research, Pfizer Inc., Groton, Connecticut 06340

Received August 24, 1998

In streptomycetes and other actinomycetes, the shikimate pathway and variations thereof play an important role in providing precursors for natural product biosynthesis. For instance, in *Streptomyces hygroscopicus* var. *ascomyceticus*, shikimic acid is converted to 3(*R*),4(*R*) dihydroxycyclohexanecarboxylic acid (DHCHC), which is subsequently used as a precursor in the biosynthesis of immunosuppressant ascomycin (Figure 1).[1] A shikimate-derived DHCHC is also used in the biosynthesis of immunosuppressant rapamycin (*Streptomyces hygroscopicus*) [2,3] and presumably FK506 *(Streptomyces tsukabaensis*).[1] None of the enzymes involved in the formation of shikimic acid in these organisms have been identified (in fact, relatively little is known concerning either the enzymes or corresponding genes of the shikimate pathway in any streptomycete).[4]

Recently, a type-II dehydroquinate dehydratase (DHQase) was cloned from *S. hygroscopicus* var. *ascomyceticus*.[5] Type-II DHQases are a family of related thermostable enzymes, that catalyze the conversion of dehydroquinic acid to dehydroshikimic acid (Figure 1).[6] Depending upon the microorganism, a type-II DHQase is involved in the quinic acid catabolic pathway, the shikimic acid biosynthetic pathway, or both pathways.[7−10] A role of the *S. hygroscopicus* var. *ascomyceticus* DHQase in providing shikimic acid, and therefore, ascomycin biosynthesis, has not been proven. Herein, we report in vitro and in vivo stereochemical analyses of the reaction catalyzed by DHQase that clearly demonstrate the role of a type-II DHQase in these processes.

It has previously been shown that the type-II DHQase of *Aspergillus nidulan*s catalyzes the formation of dehydroshikimate with loss of the *pro*-2*S* hydrogen (anti elimination).[11]

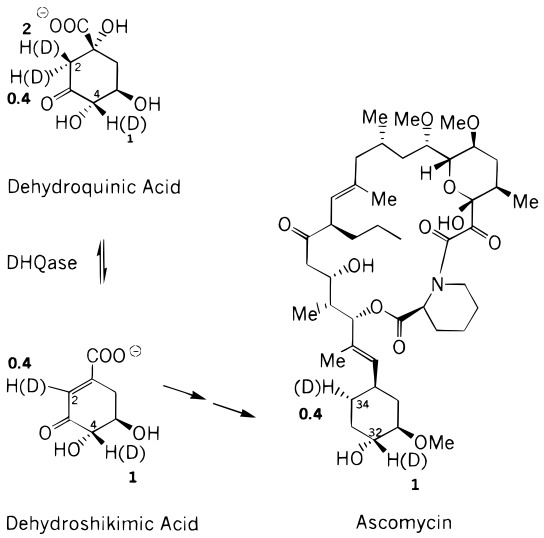

Figure 1. Role of a type-II DHQase in providing shikimic acid for ascomycin biosynthesis in *S. hygroscopicus* ssp. *ascomyceticus*. Numbers in bold reflect the approximate ratios of deuterium in these positions.

The reaction catalyzed by the type-I DHQase of *Escherichia coli* in contrast proceeds with loss of the *pro*-2*R* hydrogen (syn elimination).[11] It is unknown if these observations apply to DHQases isolated from different sources (although the strong sequence similarity within each class of DHQase would suggest that the observed stereochemical outcomes are conserved). Therefore, we prepared dehydroquinic acid differentially deuterated by treatment of dehydroquinic acid with triethylamine in D_2O.[12] Analysis of the purified dehydroquinic acid by ^2H NMR revealed three major signals at 4.3 ppm (^2H at C-4), 3.1 ppm (^2H at *pro*-2*S* position), and 2.4 ppm (^2H in the *pro*-2*R* position (Figure 1). The ratio of deuterium at these positons was 1:2:0.4, respectively (integrations were standardized against the deuterium located at C-4 as this signal was used as an internal reference for both in vivo and in vitro analysis of the stereochemical course of the DHQase reaction). Analysis by ^1H NMR revealed a doublet at 4.3 ppm, a singlet rather than a doublet at 2.4 ppm (the ratio of these peaks was approximately 1:1.3), and no clearly detectable signal remaining at 3.1 ppm, indicating almost complete exchange of the *pro*-2*S* hydrogen with deuterium.

A sample of this differentially labeled dehydroquinate (10 mg) was incubated with the recombinant DHQase from *S. hygroscopicus* (10 μg) in the presence of potassium phosphate (50 mM) at pH 7.3 overnight. As the reaction proceeded, analysis by ^2H NMR revealed a loss of the three original signals and the appearance of two signals at 4.2 and 6.4 ppm, corresponding to dehydroshikimate labeled at C-4 and C-2 in an approximate ratio of 1:0.4 ± 0.1 (Figure 1). Thus, the DHQase catalyzed formation of dehydroshikimate proceeded with loss of the *pro*-*S* deuterium (deuterium signals at 4.2 and 6.4 ppm in the ratio 1:2 would have indicated loss of the *pro*-2*R* deuterium). Analysis of the reaction product by ^1H NMR confirmed this conclusion; the ratio of

* To whom corresponding should be addressed. Tel: (804) 828 5679. Fax: (804) 827-3664. E-mail: kareynol@vcu.edu.
† Virginia Commonwealth University.
‡ Pfizer, Inc.

(1) Wallace, K. K.; Reynolds, K. A.; Koch, K.; McArthur, H. A. I.; Brown, M. S.; Wax, R. G.; Moore, B. S. *J. Am. Chem. Soc.* **1994**, *116*, 11600−11601.
(2) Paiva, N. L.; Roberts, M. F.; Demain, A. L. *J. Ind. Microbiol* **1993**, *12*, 423−428.
(3) Lowden, P. A. S.; Bohm, G. A.; Staunton, J.; Leadley, P. F. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2249−2251.
(4) Walker, G. E.; Dunbar, B.; Hunter, I. S.; Nimmo, H. G.; Coggins, J. R. *Microbiology* **1996**, *142*, 1973−1982.
(5) Florova, G.; Denoya, C. D.; Morgenstern, M. R.; Skinner, D. D.; Reynolds, K. A. *Arch. Biochem. Biophys.* **1998**, *350*, in press.
(6) Bottomley, J. R.; Clayton, C. L.; Chalk, P. A.; Kleanthous, C. *Biochem. J.* **1996**, *319*, 559−565.
(7) Hautala, J. A.; Giles, H. H.; Kinghorn, J. R. *J. Biol. Chem.* **1975**, *250*, 6008−60014.
(8) Euvernic, G. J.; Hessels, G. I.; Vrijbloed, J. W.; Coggins, J. R.; Dijkhuizen, L. *J. Gen. Microbiol.* **1992**, *138*, 2449−2457.
(9) Garbe, T.; Servos, S.; Hawkins, A. R.; Yuoung, D.; Dougan, G.; Charles, I. G. *Mol. Gen. Genet.* **1991**, *228*, 385−392.
(10) Elsmore, D. A.; Ornston, L. N. *J. Bacteriol.* **1995**, *177*, 5971−5978.
(11) Shiner, A.; Harris, J.; Kleanthous, C.; Coggins, J. R.; Hawkins, A. R.; Abell, C. *Bier. Med. Chem. Lett.* **1993**, *3*, 1399−1402.
(12) The sodium salt of 3-dehydroquinate (1 g) was dissolved in D_2O (50 mL) and the pH adjusted with sodium hydroxide to pH 7. Triethylamine was then added to adjust the pH to pH 9. The solution was stirred at room temperature for 21 days. The 3-dehydroquinate was purified on silica using ethyl acetate−methanol (95:5) as a solvent system.

10.1021/jo981712z CCC: $15.00
Published on Web 10/22/1998

signals at 4.2 and 6.4 ppm was approximately 1:1.3 consistent with dehydration reaction proceeding with retention of the *pro*-2*R* hydrogen.

The differentially labeled dehydroquinate (100 mg) was also fed to 24 h, producing cultures of *S. hygroscopicus* var. *ascomyceticus* to a final concentration 1.4 mM. After an additional 5 days of fermentation, the cells were harvested, and ascomycin (approximately 150 mg) was isolated and purified by preparative HPLC. Analysis by ^{2}H NMR revealed a signal at 3.4 and 1.6 ppm consistent with ascomycin deuterated at C32 and in the *pro*-*S* position at C34, in a ratio of 1:0.4 (Figure 1).[1] A similar pattern of labeling of ascomycin has been seen in incorporation experiments with [4-^{2}H]- and [2,5-$^2H_2$]shikimic acid.[1] These results are consistent with a processing of the labeled dehydroquinate to shikimate with a loss of deuterium label from the *pro*-2*S* position, the same stereochemical course observed in in vitro analyses of the type-II DHQase.

These stereochemical analyses thus support a role of the previously identified type-II DHQase in catalyzing the third step in the shikimic acid pathway in *S. hygroscopicus* var. *ascomyceticus*. As such, this enzyme plays a critical role in providing shikimic acid and, therefore, the DHCHC starter unit used in the biosynthesis of an important class of potent immunosuppressants. Deletion of the *dhq* gene may afford an opportunity to produce novel immunosuppressants by directed biosynthesis using analogues of DHCHC. Alternatively, increasing the levels of the DHQase within the cell may affect fermentation titers (it has already been shown that exogenously supplied shikimic acid levels stimulate rapamycin production in *S. hygroscopicus*).[13]

Acknowledgment. This work was supported by a grant from the National Science Foundation (MCB-9729528). The authors are grateful to Chris Abell at the University of Cambridge for advice on preparation and purification of differentially labeled dehydroquinate and to Dr. Neel Scarsdale for ^{2}H NMR analysis.

Supporting Information Available: Deuterium NMR spectra of the labeled dehydroquinate, dehydroshikimate, and ascomycin and a ^{1}H NMR spectrum of unlabeled dehydroquinate (4 pages).

JO981712Z

(13) Fang, A.; Demain, A. L. *Folia Microbiol.* **1995**, *40*, 607−610.