J|A|C|S
COMMUNICATIONS

Published on Web 07/24/2004

Reconstitution and Characterization of a New Desosaminyl Transferase, EryCIII, from the Erythromycin Biosynthetic Pathway

Ho Young Lee,[†] Hak Suk Chung,[‡] Chao Hang,[‡] Chaitan Khosla,*[,†,§] Christopher T. Walsh,*[,||] Daniel Kahne,*[,‡] and Suzanne Walker*[,‡]

Departments of Chemistry and Chemical Engineering, Stanford University, Stanford, California 94305, Department of Chemistry, Princeton University, Princeton, New Jersey 08544, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

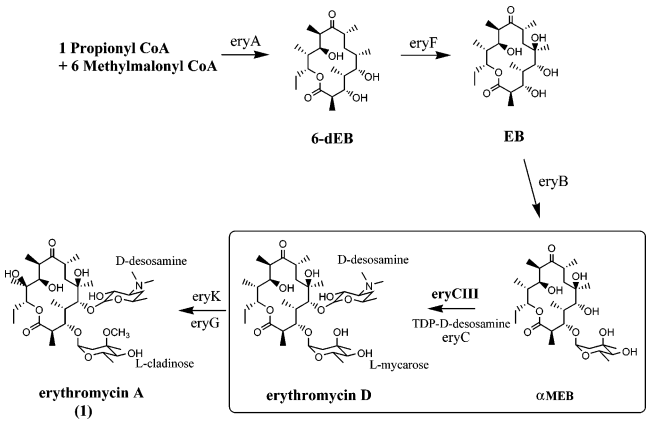
Received March 1, 2004; E-mail: ck@chemeng.stanford.edu

Erythromycin is the prototypical member of the macrolide family of antibacterial agents, which are composed of a macrocylic polyketide aglycone to which one or more deoxysugar residues are attached. Most macrolide antibacterials, including erythromycin, contain desosamine or a related amino sugar that is essential for biological activity. The emergence of resistance to erythromycin and other clinically useful macrolide antibiotics has prompted efforts to make variants of these compounds,[1] and combinatorial genetic manipulation of polyketide synthases has yielded a variety of novel aglycones.[2] Conversion of these architecturally diverse aglycones into antibacterial agents requires glycosylation with appropriate sugars. The genes encoding several desosaminyl transferases, including eryCIII from the erythromycin biosynthetic pathway[3] as well as desVII[4] and oleG1[5] from the pikromycin and oleandomycin pathways, respectively, have been sequenced, and in several cases desosaminyl transfer to macrolide substrates has been demonstrated in bacterial cells.[6] However, desosaminyl transferases have not proven straightforward to purify in active form, and their biochemical properties are not understood. Here we report the heterologous expression, purification, in vitro reconstitution, and preliminary characterization of EryCIII, laying the groundwork for detailed structural and mechanistic analysis of an important member of the desosaminyl transferase family of enzymes.

EryCIII converts α-L-mycarosyl erythronolide B into erythromycin D using TDP-D-desosamine as the glycosyl donor (Scheme 1). The eryCIII gene was cloned from *Saccharopolyspora erythraea*, which produces erythromycin, into a pET24(+) vector for expression as a C-terminal hexa-His-tagged fusion protein. Initial efforts to express the protein in the *Escherichia coli* BL21(DE3) strain under standard conditions (induction at 37 °C with 1 mM IPTG) resulted in the formation of insoluble aggregates. The outcome did not improve when expression was attempted at lower temperatures or in other host strains. However, coexpression of EryCIII with the GroEL/ES chaperone complex[7,8] was found to enhance greatly the expression of soluble EryCIII protein in BL21(DE3). Optimal expression was achieved by induction with 0.1 mM IPTG at 37 °C for 10 hours. EryCIII from the soluble fraction of a bacterial culture was loaded onto a Ni-NTA column and eluted with 200 mM imidazole. A substantial amount of GroEL coeluted with the desired protein, suggesting the presence of misfolded intermediates and necessitating an additional purification step on an anion exchange column. (Figure S1, Supporting Information) The final yield of purified EryCIII was approximately 5 mg/L.

The substrates required to assay the activity of EryCIII in vitro were obtained as follows. The donor substrate, TDP-D-desosamine,

Scheme 1. Erythromycin A (**1**) Biosynthesis Pathway



was prepared from desosamine obtained by acid hydrolysis of erythromycin B in four steps as described by Liu and co-workers.[9] αMEB (α-L-mycarosyl erythronolide B) was extracted from an EryCIII-deficient strain of *S. erythraea* grown on tryptic soy broth solid media at 30 °C for 10 days and purified via silica gel chromatography.[10] The yield was greater than 100 mg/L.

The ability of freshly purified EryCIII to transfer desosamine to αMEB was evaluated under a variety of buffer conditions. Maximum activity was detected in 50 mM phosphate buffer, pH = 8.0, containing 1 mM DTT and 5% glycerol. After a 12 h incubation of a reaction mixture containing 1 mM TDP-D-desosamine, 1 mM αMEB, and 8 μM eryCIII at 25 °C, erythromycin D was identified as the primary product detected by HPLC (Figure S2, Supporting Information). The identity of the erythromycin D product was confirmed via mass spectrometry (exact mass 703.45) and chromatographic comparison with an authentic reference standard.

To estimate the turnover number of EryCIII, changes in TDP-D-desosamine and TDP concentrations were monitored as a function of time via HPLC (Supporting Information). As shown in Figure 1, in the presence of 1 μM EryCIII and 1 mM of each substrate, more than 300 turnovers were observed in 2.5 min, indicating that the k_{cat} of EryCIII must be greater than 100 min^{-1}. This is one of the highest turnover numbers reported for an antibiotic glycosyl transferase. However, the enzyme is not particularly stable; the activity of concentrated enzyme stored overnight at 4 °C was found to be more than 3 times lower than that of freshly purified enzyme. It is possible that general instability of desosaminyl transferases is one reason activity has been difficult to detect in vitro.

A key reason for focusing on EryCIII and related enzymes is that they can potentially be used to make unnatural macrolides for use as antibacterials. Therefore, we were interested in the tolerance

[†] Department of Chemistry, Stanford University.
[‡] Princeton University.
[§] Department of Chemical Engineering, Stanford University.
[||] Harvard Medical School.

10.1021/ja048836f CCC: $27.50

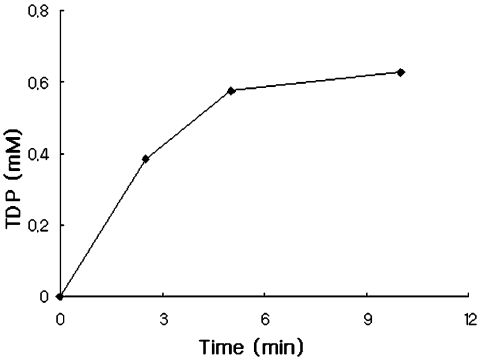
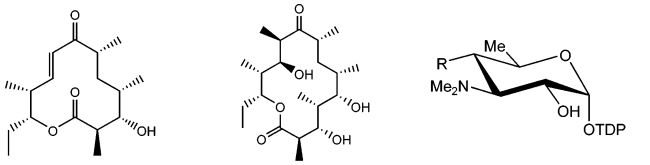


Figure 1. Conversion of αMEB and TDP-D-desosamine to erythromycin D. Assays were performed in 50 mM phosphate buffer, pH = 8.0, 1 mM DTT, and 5% glycerol in the presence of 1 mM TDP-D-desosamine and 1 mM αMEB. Substrate disappearance and product appearance was monitored by HPLC (Supporting Information).

Chart 1



of EryCIII toward alternate electrophilic and nucleophilic substrates. Assays were performed with the alternative polyketide aglycone (**1**, **2**) and TDP-sugar substrates (**4**) shown in Chart 1. Within detectable limits (as judged by HPLC and MS), neither 6-deoxyerythronolide B (**2**) nor 10-deoxymethynolide (**1**) were found to be glycosylated by EryCIII, suggesting that these compounds are at least 100-fold worse as substrates than αMEB. Furthermore, TDP-D-mycaminose, which only differs from TDP-D-desosamine at the C4 position, could not be transferred to αMEB. Thus, EryCIII appears to be quite selective for the natural nucleotide sugar donor and macrolide acceptor substrates, unlike some other antibiotic glycosyl transfersaes such as desVII,[4] oleG2,[11] ElmGT,[12] and UrdGT2,[13] which have relaxed specificity. Interestingly, EryCIII is homologous (51% identity) to DesVII, a relatively permissive (based on studies in whole cells[6]) desosaminyl transferase in the pikromycin biosynthetic pathway. Therefore, it may be possible to expand the tolerance of EryCIII to unnatural substrates by protein engineering.

In conclusion, we have reconstituted for the first time the activity of a desosaminyl transferase, EryCIII, from a macrolide biosynthetic pathway. EryCIII can be expressed and purified in active form in good yield. It has considerable specificity for its natural substrates, both sugar donor and acceptor. Structural analysis of EryCIII now appears to be feasible and, combined with protein engineering, may facilitate the use of this enzyme for combinatorial biosynthesis of new macrolide antibacterial agents.

Acknowledgment. We thank David Cane for providing a sample of 10-deoxymethynolide. This research was supported by grants from the NIH (GM067937 to C.K., GM20011 to C.T.W., AI044854 to S.W., and GM03004 to D.K.).

Supporting Information Available: Expression and purification of EryCIII. Characterization of the product of the EryCIII catalyzed reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Walsh, C. T. *Antibiotics: Actions, Origins, Resistance*; American Society of Microbiology Press: Washington, DC, 2003.

(2) Hutchinson, C. R.; McDaniel, R. *Curr. Opin. Invest. Drugs* **2001**, *2*, 1681−1690.

(3) (a) Summers, R. G.; Donadio, S.; Staver, M. J.; Wendt-Pienkowski, E.; Hutchinson, C. R.; Katz, L. *Microbiology* **1997**, *143*, 3251−3262. (b) Gaisser, S.; Bohm, G. A.; Cortes, J.; Leadlay, P. F. *Mol. Gen. Genet.* **1997**, *256*, 239−251.

(4) Xue, Y.; Zhao, L.; Liu, H. W.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12111−12116.

(5) (a) Olano, C.; Rodriguez, A. M.; Michel, J. M.; Mendez, C.; Raynal, M. C.; Salas, J. A. *Mol. Gen. Genet.* **1998**, *259*, 299−308. (b) Gaisser, S.; Reather, J.; Wirtz, G.; Kellenberger, L.; Staunton, J.; Leadlay, P. F. *Microbiology* **2000**, *36*, 391−401.

(6) Tang, L.; McDaniel, R. *Chem. Biol.* **2001**, *8*, 547−555.

(7) Barroso, J. F.; Elholm, M.; Flatmark, T. *Biochemistry* **2003**, *42*, 15158−15169.

(8) Nguyen, T. V.; Riggs, C.; Vuksanovic, D. B.; Kim, Y. S.; Carpenter, J. F.; Burghardt, T. P.; Gregersen, N.; Vockley, J. *Biochemistry* **2002**, *41*, 11126−11133.

(9) Chang, C. W.; Zhao, L.; Yamase, H.; Liu, H. W. *Angew. Chem., Int. Ed.* **2000**, *39*, 2160−2163.

(10) Hessler, P. E.; Larsen, P. E.; Constantinou, A. I.; Schram, K. H.; Weber, J. M. *Appl. Microbiol. Biotechnol*. **1997**, *47*, 398−404.

(11) Doumith, M.; Legrand, R.; Lang, C.; Salas, J. A.; Raynal, M. C. *Mol. Microbiol.* **1999**, *34*, 1039−1048.

(12) Blanco, G.; Patallo, E. P.; Bra×f7ña, A. F.; Trefzer, A.; Bechthold, A.; Rohr, J.; Méndez, C.; Salas, J. A. *Chem. Biol.* **2001**, *8*, 253−263.

(13) Hoffmeister, D.; Dräger, G.; Ichinose, K.; Rohr, J.; Bechthold, A. *J. Am. Chem. Soc.* **2003**, *125*, 4678−4679.

JA048836F