Biosynthesis of Mycarose: Isolation and Characterization of Enzymes Involved in the C-2 Deoxygenation

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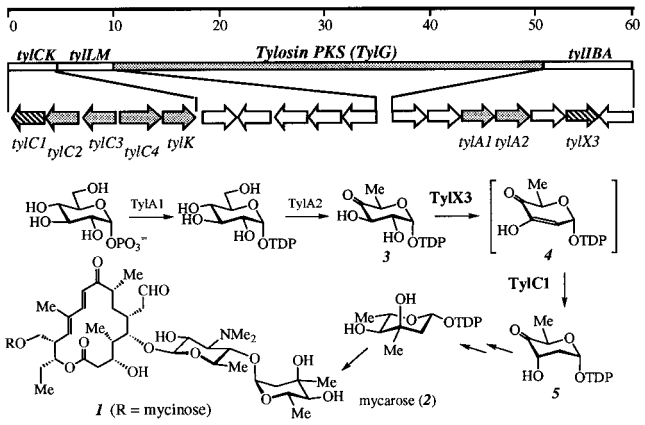
The breaking of carbon−oxygen bonds is a fundamentally important reaction, essential for many physiological processes ranging from DNA synthesis to energy metabolism. It is thus not surprising that a diverse array of mechanisms have evolved to facilitate biological C−O bond scission.[1] One of the more interesting examples of such a mechanism involves the radical-based 3-deoxygenation step during ascarylose biosynthesis.[2,3] In this system, the 3-OH of CDP-4-keto-6-deoxy-D-glucose is irreversibly excised by a collaborative catalysis between a pyridoxamine 5′-phosphate (PMP)-dependent [2Fe-2S]-containing enzyme (E_1)[4] and a NADH-dependent reductase (E_3).[5] Since deoxygenation of common sugars appears to be a seemingly simple yet effective modification adopted by nature to generate carbohydrate structural permutations, the study of deoxysugar biosynthesis offers an excellent opportunity to learn nature's diverse strategies for breaking different C−O bonds.[1]

Due to the successful characterization of the novel pathway and enzymatic activities associated with C-3 deoxygenation,[2,3] increasing attention is presently being shifted to the C-2 deoxygenation event involved in the biosynthesis of 2,6-dideoxyhexoses, which are commonly found in secondary metabolites.[2b,c] This is certainly the case for mycarose (**2**), a 2,6-dideoxy sugar with a C-3 methyl branch that is one of the three sugar components of the macrolide antibiotic tylosin (**1**), produced by *Streptomyces fradiae*. Early genetic studies yielded the entire gene cluster responsible for the biosynthesis of tylosin (Scheme 1)[6] and also revealed that the genes involved in the biosynthesis of mycarose are in the *tylCK* and *tylIBA* regions. Recently, we have sequenced these two regions, from which 12 open reading frames (ORFs) were identified.[7,8] As shown in Scheme 1, the genes believed to participate in the formation of mycarose are assigned on the basis of sequence similarities to other sugar biosynthetic genes, particularly those derived from the biosynthetic gene cluster of erythromycin,[9] which also contains a mycarose derivative (cladinose) as one of its appended sugars.

Scheme 1



While removal of the 2-OH in the mycarose pathway may follow a mechanism analogous to that for C-3 deoxygenation catalyzed by E_1 and E_3,[2b,c] the absence of the homologous E_1 and E_3 genes in the tylosin and erythromycin clusters has led to an alternative dehydration mechanism (see Scheme 1).[9,10] In a study of the biosynthesis of granaticin and granaticin B, which contain a 2,6-dideoxy-D-hexose moiety 1,4-linked to a benzoisochromane quinone chromophore, Draeger et al. reported the identification of a dehydratase (Gra Orf 27) and a reductase (Gra Orf26) that could convert **3** to a stable TDP-4-keto-2,6-dideoxyglucose product.[11] Their data provided the first biochemical evidence validating the proposed dehydration mechanism. Here, we report the findings of our own investigations into the mechanism of C-2 deoxygenation in the biosynthesis of mycarose (**2**). Not only do our results corroborate the conclusion reached by Draeger et al., but more importantly, our biochemical characterization of the enzymes involved in C-2 deoxygenation also offers significant insights into the mode of their catalyses.

As depicted in Scheme 1, the *tylX3* gene downstream of the *tylIBA* region has been assigned as the dehydratase responsible for the C-2 deoxygenation event, while *tylC1* within the *tylCK* region has been speculated to be the reductase gene. To obtain the TylX3 and TylC1 proteins, their respective coding regions were amplified by the polymerase chain reaction (PCR). The *tylX3* gene was cloned into the expression vector pET-28b(+) with a His_6-tag at the *N*-terminus, whereas *tylC1* was cloned into pET-24b(+) with a His_6-tag at the *C*-terminus. Both proteins were purified to near homogeneity using Ni−NTA resin (Qiagen) and were found to be stable in phosphate buffers containing 15% (v/v) glycerol. The identities of these proteins were confirmed by *N*-terminal amino acid analysis.[12] Further analysis using size-exclusion chromatography (Superdex 200) revealed that TylX3 exists as a homodimer with a subunit molecular weight of 55 kDa, and TylC1 consists of a single polypeptide with molecular

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(1) Johnson, D. A.; Liu, H.-w. In *The Biology-Chemistry Interface: A Tribute to Koji Nakanishi*; Cooper, R., Snyder, J. D., Eds.; Marcel Dekker: New York; 1999, in press.

(2) (a) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol*. **1994**, *48*, 223−256. (b) Kirschning, A.; Bechthold, A. F.-W.; Rohr, J. In *Bioorganic Chemistry Deoxysugars, Polyketides & Related Classes: Synthesis, Biosynthesis, Enzymes*; Rohr, J., Ed.; Springer: Berlin, 1997; pp 1−84. (c) Johnson, D. A.; Liu, H.-w. In *Comprehensive Chemistry of Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Pergamon: New York, 1999; Vol. 3, pp 311−365.

(3) (a) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 12177−12178. (b) Johnson, D. A.; Gassner, G. T.; Bandarian, V.; Ruzicka, F. J.; Ballou, D. P.; Reed, G. H.; Liu, H.-w. *Biochemistry* **1996**, *35*, 15846−15856.

(4) (a) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 7539−7540. (b) Lei, Y.; Ploux, O.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4643−4654.

(5) (a) Miller, V. P.; Thorson, J. S.; Ploux, O.; Lo, S. F.; Liu, H.-w. *Biochemistry* **1993**, *32*, 11934−11942. (b) Chen, X. M. H.; Ploux, O.; Liu, H.-w. *Biochemistry* **1996**, *35*, 16412−16420.

(6) (a) Baltz, R. H.; Seno, E. T. *Antimicrob. Agents Chemother*. **1981**, *20*, 214−225. (b) Baltz, R. H.; Seno, E. T. *Annu. Rev. Microbiol*. **1988**, *42*, 547−574.

(7) Chen, H.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 9951−9952.

(8) It should be pointed out that the *tylIBA* and *tylLM* segments of the *tyl* cluster have also been sequenced by Cundliffe and co-workers (Merson-Davies, L. A.; Cundliffe, E. *Mol. Microbiol*. **1994**, *13*, 349−355. Gandecha, A. R.; Large, S. L.; Cundliffe, E. *Gene* **1997**, *184*, 197−203. Fish, S. A.; Cundliffe, E. *Microbiology* **1997**, *143*, 3871−3876. Bulter, A. R.; Bate, N.; Cundliffe, E. *Chem. Biol*. **1999**, *6*, 287−292).

(9) (a) Gaisser, S.; Bohm, G. A.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet*. **1997**, *256*, 239−251. (b) Salah-Bey, K.; Doumith, M.; Michel, J.-M.; Haydock, S.; Cortés, J.; Leadlay, P. F.; Raynal, M.-C. *Mol. Gen. Genet*. **1998**, *257*, 542−553. (c) Summers, R. G.; Donadio, S.; Staver, M. J.; Wendt-Pienkowski, E.; Hutchinson. C. R.; Katz, L. *Microbiology* **1997**, *143*, 3251−3262.

(10) (a) Thorson, J. S.; Lo, S. F.; Liu, H.-w.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1993**, *115*, 5827−5828. (b) Johnson, D. A.; Liu, H.-w. *Curr. Opi. Chem. Biol*. **1998**, *2*, 642−649. (c) Hallis, T. M.; Liu, H.-w. *Acc. Chem. Res*. **1999**, *32*, 579−588.

(11) Draeger, G.; Park, S.-H.; Floss, H. G. *J. Am. Chem. Soc*. **1999**, *121*, 2611−2612.

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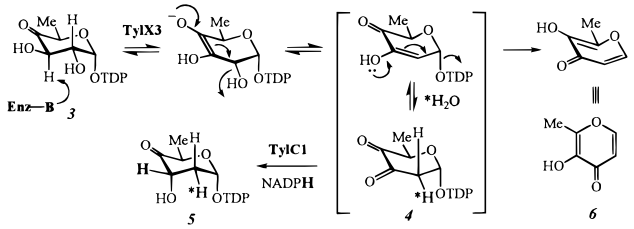
Scheme 2



weight of 36 kDa.[13] The UV−visible spectra of both proteins were transparent above 300 nm. However, inductively coupled plasma (ICP) analysis showed the presence of 0.8 equiv of Zn^{2+} bound per TylX3 monomer. Prolonged dialysis of TylX3 against 5 mM 1,10-phenanthroline led to a total loss of activity, further implicating a role for Zn^{2+} in catalysis.[14] ICP analysis of the 1,-10-phenanthroline-treated enzyme showed the absence of Zn^{2+}, which correlates well with the loss of enzyme activity. The specific activity of the native enzyme was also found to increase about 8-fold when it was dialyzed against 5 mM $ZnSO_4$.

When TylX3 was incubated with the substrate **3**, which was prepared from TDP-D-glucose using the 4,6-dehydratase RfbB[15] from the rhamnose pathway, consumption of substrate and the concomitant formation of TDP and a new product were detected by HPLC (see Scheme 2).[16] This new compound was isolated from an ethyl acetate extract and was identified as maltol (**6**) by comparing its ^{1}H NMR spectrum with that of an authentic sample (from Aldrich). A similar observation was also noted by Draeger et al. in which formation of maltol by Gra Orf27 had been suggested as a result of degradation of the expected dehydration product **4**.[11] Interestingly, when **3** was incubated with TylX3 together with TylC1 and NADPH, a new product was detected by HPLC.[17,18] This product was purified by gel filtration chromatography (Bio-Rad P2, extra fine, 1 × 150 cm, elution with 10 mM KCl at 8 mL/h) and identified as TDP-2,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**5**) by ^{1}H and ^{13}C NMR.[19] Again, a reductase (Gra Orf26) was also required to afford a stable sugar product, as was observed by Draeger et al.[11]

When the incubation was carried out in buffer prepared with D_2O, deuterium incorporation was found at the C-2 equatorial position in **5**.[19] These results, while clearly indicating that displacement of the 2-OH with a solvent hydrogen proceeds with retention of configuration, also imply that the hydride is transferred from NADPH to the C-3 position of **4**. It should be noted that the C-3 hydroxyl group in **5** is axial, as revealed by the coupling constants for H-3 (J_{H2a-H3} = 3.5, J_{H2e-H3} = 3.0 Hz), which is also consistent with the lack of nuclear Overhauser effect (NOE) between H-3 and H-5. This assignment is in contrast to the Gra Orf26 product isolated by Draeger et al.[11]

Upon reduction of **4**, whose instability is highlighted by its ready loss of TDP, the more stable product **5** is formed, which is sufficiently stable to enable purification and characterization. It is tempting to speculate whether some form of protein−protein interaction leading to substrate channeling occurs in vivo to maximize the utilization of the unstable intermediate **4** by TylC1. However, attempts to detect interactions between TylX3 and TylC1 both in vivo using the yeast two-hybrid system[20] and in vitro using the gel filtration chromatography were not fruitful.

Not only does TylX3 have interesting catalytic properties, but of further significance is that its deduced sequence shows a high degree of homology to those of *eryBVI* in the erythromycin pathway,[9] *lanS* from the landomycin pathway,[21] *orf23* in the vancomycin pathway,[22] *snoH* in the nogalamycin pathway,[23] *dnmT* from the doxorubicin pathway,[24] and *orf27* in the granaticin pathway.[25] Thus, it is plausible that these enzymes constitute a small family of Zn^{2+}-dependent dehydratases involved in C-2 deoxygenation for the generation of the 2,6-dideoxyhexose moieties in their respective systems. Although TylC1 and Gra Orf26 both are typical NADPH-dependent keto-reductases, the lack of any significant sequence similarity between the corresponding genes, and the fact that the product of TylC1 catalysis is stereochemically distinct from that of Gra Orf 26,[11] seem to indicate that these two enzymes have different evolutionary backgrounds.

In conclusion, these results confirm the mode of C-2 deoxygenation in the biosynthesis of 2,6-dideoxyhexoses. Our biochemical characterization of TylX3 and TylC1 clearly revealed that nature has opted for a metal-assisted dehydration/reduction sequence to remove a β-OH (at C-2) from a ketohexose (4-keto) precursor. A similar β-elimination mechanism has also been found for C-6 deoxygenation in the biosynthesis of 6-deoxyhexoses, in which the expulsion of the 6-OH (β-OH) from a 4-ketohexose intermediate generates a 4-keto-6-deoxyhexose product (such as **3**).[1,2,26] Clearly, a more elaborate mechanism has been evolved to remove an α-OH from a ketohexose precursor, as in the case of C-3 deoxygenation. Taken together, these differences are a testament to the evolutionary diversity of biological C−O bond cleavage events, and as more sugar biosynthetic systems become characterized, it will be interesting to see what other novel deoxygenations may be discovered.

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(12) After thrombin digestion to remove the His_6 tag, the *N*-terminal amino acid sequencing confirmed that the first 10 residues of TylX3 (AHSSATAGPQ) and TylC1 (SGMYVQLGR) are identical to the respective translated *tylX3* and *tylC1* sequences, except for the deletion of the first methionine residue in both cases.

(13) The calculated molecular mass for TylX3 is 55 795 Da, and that for TylC1 is 36 920 Da.

(14) The actual role of Zn^{2+} in TylX3 catalysis must await further investigation.

(15) Romana, L. K.; Santiago, F. S.; Reeves, P. R. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 846−852.

(16) The incubation mixture was loaded on an Adsorbosphere SAX column (5 μm, 4.6 × 250 mm), and a linear gradient from 140 to 320 mM potassium phosphate buffer (pH 3.6) over 20 min was used to elute the reaction products (monitored at 278 nm). Under these conditions, the retention times were 4.8 min for maltol (**6**), 13.0 min for substrate **3**, and 17.6 min for TDP.

(17) A preparative incubation contained the RfbB product (**3**, 14.0 mg, 23.7 μmol) and NADPH (21.7 mg, 26.1 μmol) in 1.5 mL of 100 mM potassium phosphate buffer (pH 7.5). The reaction was initiated by the addition of TylX3 (6.5 nmol) and TylC1 (10.5 nmol). Due to the instability of the TylX3 product, an excess of TylC1 was used. The reaction was incubated at room temperature and was followed by monitoring the consumption of NADPH at 340 nm. The reaction was usually complete within 2 h.

(18) The same HPLC conditions as described in ref 16 were used. The retention time for product **5** was 13.8 min.

(19) ^{1}H NMR (D_2O, hydrated form): δ 1.05 (3H, d, J = 6.5 Hz, 5-Me), 1.80 (3H, s, 5″-Me), 1.97 (1H, ddd, J = 15.0, 3.0, 1.5, 2-H_{eq}), 2.09 (1H, dq, J = 15.0, 3.5, 2-H_{ax}), 2.18−2.27 (2H, m, 2′−H), 3.69 (1H, dd, J = 3.0, 3.5, 3-H), 3.96−4.04 (3H, m, 4′-H, 5′-H), 4.18 (1H, q, J = 6.6, 5-H), 4.47−4.48 (1H, m, 3′-H), 5.43 (1H, m, 1-H), 6.16 (1H, t, J = 6.6, 1′-H), 7.58 (1H, s, 6″-H). ^{13}C NMR (D_2O, hydrated form): δ 11.5, 11.6, 33.7 (d, J = 8.4 Hz), 38.0, 64.7 (d, J = 5.2 Hz), 65.6, 68.9, 70.2, 84.6, 85.0 (d, J = 8.6 Hz), 91.8, 93.5 (d, J = 6.0 Hz), 111.6 (d, J = 2.9 Hz), 137.3, 151.6, 166.5. ^{31}P NMR (D_2O): δ −10.7 (d, J = 21.9 Hz), −12.9 (d, J = 21.9 Hz).

(20) (a) Fields, S.; Song, O.-k. *Nature* **1989**, *340*, 245−246. (b) Vojtek, A. B.; Hollenberg, S. M.; Cooper, J. A. *Cell* **1993**, *74*, 205−214.

(21) Westrich, L.; Domann, S.; Faust, B.; Bedford, D.; Hopwood, D. A.; Bechthold, A. *FEMS Microbiol. Lett.* **1999**, *170*, 381−387.

(22) van Wageningen, A.; Kirkpatrick, P.; Williams, D.; Harris, B.; Kershaw, J.; Lennard, N.; Jones, M.; Jones, S.; Solenberg, P. *Chem. Biol.* **1998**, *3*, 155−162.

(23) Torkkell, S.; Ylihonko, K.; Hakala, J.; Skurnik, M.; Mantsala, P. *Mol. Gen. Genet.* **1997**, *256*, 203−209.

(24) Scotti, C.; Hutchinson, C. R. *J. Bacteriol.* **1996**, *178*, 7316−7321.

(25) Ichinose, K.; Bedford, D. J.; Tornus, D.; Bechthold, A.; Bibb, M. J.; Revill, W. P.; Floss, H. G.; Hopwood, D. A. *Chem. Biol.* **1998**, *5*, 647−659.

(26) Glaser, L.; Zarkowsky, R. In *The Enzymes*; Boyer, P., Ed.; Academic Press: New York, 1971; Vol. 5, pp 465−480. (b) Gabriel, O. In *Carbohydrates in Solution*; Gould, R., Ed.; Advances in Chemistry Series 117, American Chemical Society: Washington, DC, 1973; pp 387−410. (c) Snipes, C. E.; Brillinger, G.-U.; Sellers, L.; Mascaro, L.; Floss, H. G. *J. Biol. Chem.* **1977**, *252*, 8113−8117. (d) Yu, Y.; Russell, R. N.; Thorson, J. S.; Liu, L.-d.; Liu, H.-w. *J. Biol. Chem.* **1992**, *267*, 5868−5875.