Expression, Purification, and Characterization of TylB, an Aminotransferase Involved in the Biosynthesis of Mycaminose

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Received April 16, 1999

Amino sugars are ubiquitously found in nature as constituents of glycoproteins, glycolipids, and a great variety of secondary metabolites.[1] They have been shown to contribute to the physiological functions of many glycoconjugates, and play pivotal roles in determining the efficacy and specificity of numerous clinically relevant natural products.[2] For example, several of the lipopolysaccharide-based amino sugars are known to define the immunogenic characteristics of a specific bacterial strain.[1e,3] In addition, the common hydroxy/amino motif of the amino sugars in various aminoglycoside antibiotics has been demonstrated to be the key element responsible for the interaction between the antibiotics and their DNA/RNA targets.[4]

Biosynthetically, the amino sugars have been proposed to be derived from the corresponding keto sugars via a pyridoxal 5′-phosphate (PLP)-dependent transamination reaction.[5] Recent genetic as well as phenotypic complementation studies of the biosynthesis of *O*-antigens and many sugar-containing secondary metabolites have accumulated a vast amount of information pertaining to unusual sugar formation.[6] On the basis of the modest residue homology of the amino sugar biosynthetic genes to other available PLP-dependent enzymes, a number of these genes have been speculated to encode the aminotransferases for making amino sugars.[7] In particular, studies of the biosynthesis of tylosin (**1**),[8] a macrolide antibiotic that contains the 3,6-dideoxy-3-dimethylamino sugar mycaminose (**2**), has led to the assignment of *tylB* as the likely candidate encoding the requisite C-3 aminotransferase.[7,9,10] The tentative assignment of *tylB* along with other genes in the *tylLM* and *tylIBA* regions has allowed the postulation of a possible route for mycaminose biosynthesis as shown in Scheme 1.[6,9,10] However, like most sugar biosynthetic genes, the corresponding gene products have never been isolated and characterized, and hence, the assignment of the actual catalytic role of *tylB* and other postulated aminotransferase genes awaits more concrete validation.

To determine the function of TylB, the *tyl*B gene was amplified by polymerase chain reaction (PCR) and cloned into the expression vector pET24b(+). The recombinant plasmid, pHC-28, was used to transform *Esherichia coli* BL21(DE3), and the resulting cells were grown at 30 °C in LB medium with induction by isopropyl β-D-thiogalactoside (IPTG, 0.1 mM) to produce C-terminal His-tagged TylB. This enzyme was purified to near homogeneity by a Ni-NTA column (Qiagen), and its identity was confirmed by *N*-terminal amino acid sequencing.[11] Judging from a M_r of 84.1 KDa estimated by gel filtration and a calculated mass of 42023 Da based on the translated sequence, TylB was determined to exist as a homodimer. Surprisingly, the purified TylB showed no absorption above 300 nm and was evidently devoid of a PLP coenzyme. Attempts to reconstitute TylB with PLP under various denaturation/renaturation conditions proved futile as the reconstituted sample remained to be transparent above 300 nm. The failure to detect a B_6 coenzyme in the wild-type as well as the reconstituted TylB was discouraging and raised doubt about its assigned catalytic role. Thus, a comprehensive test of the catalytic activity of TylB was undertaken to critically determine whether the early assignment of TylB as the sought after C-3 aminotransferase, based on sequence homology, was with or without merit.

As shown in Scheme 1, the predicted substrate of TylB is a TDP-6-deoxy-3-keto sugar **4** that has recently been synthesized.[12] However, due to its relatively low hydrolytic stability, a large supply of this substrate was not readily available for a thorough analysis of TylB activity. Hence, while initial reconstitution experiments utilized synthetic samples of **4**, an alternative strategy that would obviate the need for **4** was more desirable for studying the function of TylB. Considering the fact that the putative transamination product, **5**, is more stable, its chemical synthesis is comparatively much less demanding,[10] and the enzyme-catalyzed transamination is a reversible process, the activity of TylB may be more conveniently assayed in the reverse direction (**5** → **4**). To test the feasibility of this approach, the amino sugar **5**, prepared in a separate experiment,[10] was incubated with a catalytic amount of TylB and PLP in the presence of α-ketoglutarate (Scheme 2). To our delight, a new product was detected at 267 nm by HPLC using an Adsorbsphere SAX column (4.6 × 250 mm). Baseline separation of the product (retention time =

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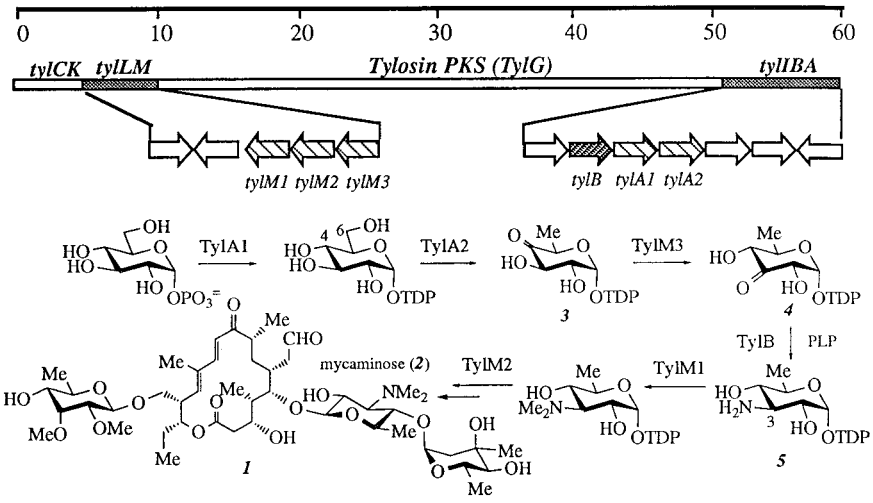
10.1021/ja991213v CCC: $18.00
Published on Web 07/16/1999

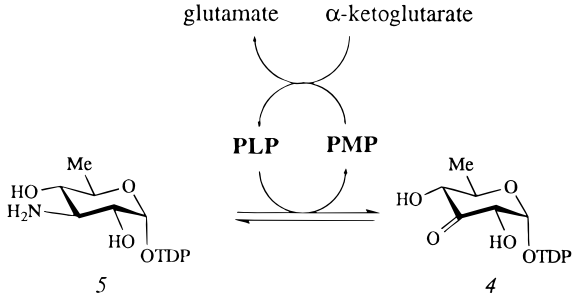
Scheme 1



15.7 min) from the substrate (retention time = 6.6 min) was achieved using a linear gradient from 50 to 140 mM potassium phosphate buffer (pH 3.5) with a flow rate of 1.0 mL/min over 20 min. The purified product was fully characterized by ^{1}H and ^{13}C NMR,[13,14] and its spectral data were identical with those of **4**.[12] These results confirmed that TylB is indeed the desired aminotransferase that catalyzes the C-3 transamination step in the biosynthesis of mycaminose (**2**).

It is worth mentioning that the conversion of **5** to **4** could be driven to completion by the addition of excess α-ketoglutarate, and pyruvate is an alternative, albeit less efficient, amino acceptor. It was also found that the 4-keto sugar **3** is not a substrate for TylB,[15] and pyridoxamine 5′-phosphate (PMP) can effectively substitute PLP in TylB catalysis. More importantly, TylB recovered from the above incubation mixture exhibited an absorption spectrum typical for a PLP-containing enzyme, and was fully active in the absence of exogenous PLP/PMP. Apparently, the presence of both PLP and the necessary substrates is essential for coenzyme reconstitution of TylB and was the missing piece in our early reconstitution efforts. While a full understanding of its catalytic properties must await further characterization, a recent gene replacement experiment indicates TylB can also recognize and process a 3-keto-sugar substrate lacking the 4-hydroxyl group.[16]

Scheme 2



The results summarized herein provide, for the first time, biochemical evidence confirming the role of TylB as the aminotransferase required for the C-3 transamination step in the biosynthesis of mycaminose. It should be noted that isomerization of **3** to **4** was previously reported to occur during the purification of **3** by Dowex-1 ion exchange chromatography, and thus, such a keto−enol tautomerization was speculated to be a nonenzymatic process.[17] However, in view of our findings that sugar **3** is not a substrate for TylB and sugar **4** is the only product from **5** in the TylB-catalyzed transformation, the conversion of **3** to **4** in the mycaminose pathway must be a necessary enzymatic step mediated by a tautomerase. Since the deduced sequence of *tylB* shows significant homology to many genes believed to be involved in the biosynthesis of unusual sugars,[7] the fact that TylB has now been fully established as a PLP-dependent aminotransferase strongly indicates a similar role for these proteins in their respective biosynthetic pathways.

Acknowledgment. This work was supported in part by National Institutes of Health Grants GM35906 and GM54346 (to H.-w.L.), the Deutsche Forschungsgemeinschaft, and the Fonds der Chemischen Industrie (to R.R.S.).

JA991213V

(13) To characterize this product, a preparative scale incubation was carried out in which 14.2 nmol of **5**, 0.2 mmol of α-ketoglutarate, 0.7 nmol of PLP, and 0.7 nmol of TylB were incubated in 2 mL of 100 mM potassium phosphate buffer, pH 7.5, at 24 °C for 3 h. The enzyme was removed using a Centricon-10 and the filtrate was separated on HPLC using a C_{18} column (Econosil, 10 μ, 10 × 250 mm). The desired product was eluted isocratically by 4% CH_3-CN in 500 mM $Et_3NH{\cdot}HCO_3$ solution (flow rate = 1.5 mL/min) with a retention time of 22 min. The collected sample was treated with ion exchanger (Amberlite IR 120, Na^+ form), filtered, and lyophilized. The product was subjected to spectroscopic analysis.

(14) Spectral data of **4**: ^{1}H NMR (D_2O) δ 1.21 (3H, d, J = 5.1 Hz, 5-Me), 1.71 (3H, s, 5″-Me), 2.13−2.19 (2H, m, 2′-Hs), 3.93−4.04 (5H, m, 4-H, 5-H, 4′-H, 5′-Hs), 4.39−4.41 (1H, m, 2-H), 4.44−4.49 (1H, m, 3′-H), 5.66 (1H, dd, J = 7.3, 4.4 Hz, 1-H), 6.12 (1H, t, J = 7.0 Hz, 1′-H), 7.53 (1H, s, 6″-H); ^{13}C NMR (D_2O) δ 12.0 (5″-Me), 18.1 (C-6), 38.6 (C-2′), 64.9 (C-5′, d, J = 3.7 Hz), 70.6 (C-3′), 72.5 (C-5), 74.9 (C-2, d, J = 9.3 Hz), 77.5 (C-4), 85.0 (C-1′), 85.7 (C-4′, d, J = 9.0 Hz), 97.9 (C-1, d, J = 6.1 Hz), 112.1 (C-5″), 137.8 (C-6″), 152.2 (C-2″), 167.0 (C-4″), 206.8 (C-3); ^{31}P NMR (D_2O) δ −8.46 (d, J = 20.8 Hz), −10.64 (d, J = 20.8 Hz). The ribose hydrogens are denoted with a (′) and those of thymine are denoted with a (″).

(15) Sugar **3** was prepared enzymatically from TDP-D-glucose using purified RfbB, a TylA2 equivalent from *Salmonella enterica* LT2, which catalyzes the same reaction as TylA2 in the rhamnose pathway (Romana, L. K.; Santiago, F. S.; Reeves, P. R. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 846−852).

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