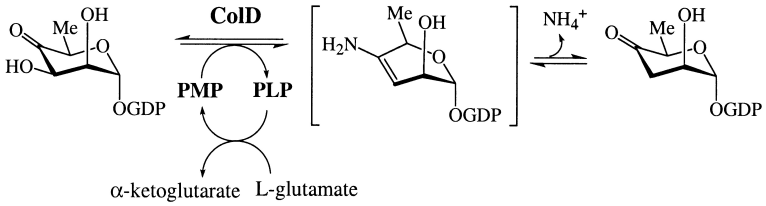
Communication

The Biosynthesis of GDP-l-Colitose: C-3 Deoxygenation Is Catalyzed by a Unique Coenzyme B-Dependent Enzyme

Noelle Beyer, Jenefer Alam, Tina M. Hallis, Zhihong Guo, and Hung-wen Liu

J. Am. Chem. Soc., **2003**, 125 (19), 5584-5585• DOI: 10.1021/ja030088r • Publication Date (Web): 19 April 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 04/19/2003

The Biosynthesis of GDP-L-Colitose: C-3 Deoxygenation Is Catalyzed by a Unique Coenzyme B_6-Dependent Enzyme

Noelle Beyer, Jenefer Alam, Tina M. Hallis,† Zhihong Guo,‡ and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712, and Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

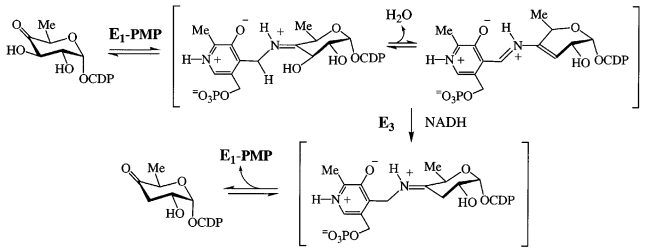
Received February 7, 2003; E-mail: h.w.liu@mail.utexas.edu

L-Colitose (**1**) is a 3,6-dideoxyhexose found in the *O*-antigen of gram-negative lipopolysaccharides.[1] Early biosynthetic studies of 3,6-dideoxysugars have shown that deoxygenation at C-3 is accomplished by an elegant mechanism involving a complementary pair of enzymes, E_1, a pyridoxamine 5′-phosphate (PMP)-dependent iron−sulfur-containing enzyme,[2] and E_3, a [2Fe−2S]-containing flavoprotein reductase.[3] As depicted in Scheme 1, the overall reaction proceeds via a dehydration/reduction mechanism, which is a unique process leading to the formation of this class of unusual sugars.[4] However, unlike other 3,6-dideoxyhexoses, which are all derived from CDP-D-glucose, the precursor of L-colitose is GDP-D-mannose.[5] Clearly, a distinct set of enzymes must have been adopted for colitose biosynthesis. Whether the C-3 deoxygenation in the colitose pathway follows a route analogous to those found in other glucose-derived 3,6-dideoxysugars has received much attention in our laboratory over the past few years.

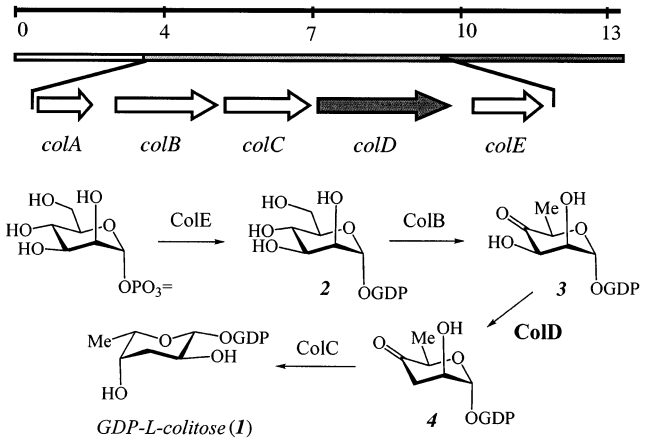
In the course of these studies, we cloned and sequenced the L-colitose biosynthetic gene cluster from *Yersinia pseudotuberculosis* IVA[6] and identified five open reading frames (Scheme 2). Function assignment of the genes in this cluster based on sequence comparison with known genes in the databank suggested a biosynthetic pathway similar to those established for other 3,6-dideoxysugars with two apparent disparities: the lack of an E_3 equivalent for the C-3 deoxygenation step (**3** → **4**), and the lack of a specific epimerase to invert the configuration at C-5 (**4** → **1**). In addition, while the translated *colD* gene sequence shows moderate homology to that of the E_1 gene (*ddhC*/*ascC*)[7] of the ascarylose pathway (27% identity and 42% similarity), it lacks the putative iron−sulfur binding motif found in the E_1 sequence. To verify the assigned functions of these genes, the encoded proteins, ColB and ColD, were expressed and purified. Reported herein are the results of our mechanistic studies of ColD, whose mode of action for catalyzing the C-3 deoxygenation in the biosynthesis of GDP-L-colitose (**1**) deviates significantly from the E_1/E_3 type of reaction and represents a new paradigm for coenzyme B_6-mediated deoxygenation.

To determine the function of ColD, the *colD* gene was cloned into a pET-28b vector, and the resulting construct, pNB002, was used to transform *Escherichia coli* HMS174(DE3) cells (Novagen). The expressed protein, containing an N-terminal His_6 tag, was purified to near-homogeneity using Ni-NTA resin (Qiagen).[8] Because no absorption was apparent above 300 nm, ColD, which is presumably coenzyme B_6-dependent based upon its homology with E_1, was isolated primarily as an apoprotein. It should be noted that pyridoxal 5′-phosphate (PLP) is the common coenzyme form for coenzyme B_6-dependent enzymes, where PLP is covalently bound to the protein by forming a Schiff base with a highly conserved lysine residue in the active site. However, this conserved lysine is replaced by a histidine in ColD (H192). Such a substitution also occurs in E_1 (AscC) of the ascarylose pathway (H222) and has been considered a trait unique to PMP-linked enzymes.[4,9] Thus, the fact that ColD is devoid of absorption characteristic for an internal PLP-aldimine adduct is consistent with its proposed role as a PMP-dependent E_1 equivalent, because the PMP cofactor should not be covalently bound to ColD.

Scheme 1



Scheme 2

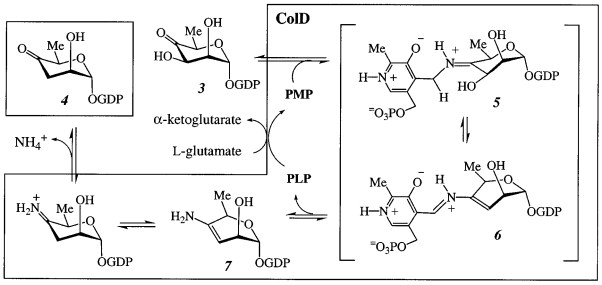


The hypothesis that ColD is a PMP-dependent enzyme was also supported by our observation that turnover occurred upon the incubation of ColD with substrate **3** in the presence of excess PMP, but not in the presence of PLP. However, the rate of product formation increased by more than 40-fold when L-glutamate was included in the PLP incubation.[10] Because α-ketoglutarate was identified as a byproduct under the above assay conditions, it became clear that ColD functions as a transaminase and that it recognizes both PMP and PLP. The aforementioned rate enhancement by L-glutamate may simply reflect the fact that the conversion of PLP to PMP via the transamination recycling reaction is more facile than a direct physical exchange of PLP in the active site with excess PMP in the solution. Further experiments showed that

† Current address: Pan Vera Corp., Discovery Center, 501 Charmany Drive, Madison, WI 53719.
‡ Current address: Department of Chemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong.

10.1021/ja030088r CCC: $25.00

Scheme 3



L-glutamate is a better amino donor than L-glutamine (~50% activity) and L-alanine (<5% activity), while D-glutamate and L-aspartate are inactive as cosubstrates. Similar results have also been noted for many aminotransferases,[11] thus substantiating an analogous aminotransferase activity for ColD.

On the basis of these findings, the first step of ColD catalysis likely involves the conversion of PLP to PMP via an amino transferring reaction. Subsequent large scale incubation allowed the isolation of the turnover product,[12] which was identified by spectral analyses as the desired 3-deoxygenated sugar **4**.[13] Clearly, ColD is the C-3 deoxygenase which functions without a partner, in contrast to the C-3 deoxygenation reaction performed by the paired E_1/E_3 enzymes.[14]

Having established the capability of ColD to function as a transaminase as well as a deoxygenase, we can now propose a mechanism for its catalysis as shown in Scheme 3. The first half-reaction involves the conversion of PLP to PMP, a prelude to transamination. The second half-reaction involves nitrogen transfer from PMP to the sugar substrate, which is initiated by the formation of a Schiff base (**5**) between PMP and **3**, followed by 1,4-dehydration to eliminate the 3-hydroxyl group. Hydrolysis of the $\Delta^{3,4}$-amino-mannoseen intermediate (**6**) regenerates PLP, and the resulting enamine sugar (**7**) then undergoes tautomerization followed by hydrolysis to give the 4-keto-3,6-dideoxymannose product (**4**), releasing ammonia in the process. The stereospecific incorporation of a deuterium at 3-H_{eq} of **4** when the reaction was conducted in deuterated buffer indicated that tautomerization of **7** must be an enzyme-mediated event. Such a mechanism resembles those of PLP-dependent serine/threonine dehydratases.[15] However, in these enzymes, the nitrogen atom of the enamine moiety of the dehydration product is part of the substrate, whereas in the ColD reaction, the nitrogen of the enamine sugar (**7**) is derived from the PMP coenzyme. Hence, ColD catalysis involves not only a dehydration reaction because a transamination step is necessary to reload the amino group on the coenzyme after each catalytic cycle.

In summary, C-3 deoxygenation catalyzed by ColD is distinct from the E_1/E_3 deoxygenation reaction in two major ways. First, in the biosynthesis of GDP-L-colitose, C-3 deoxygenation is catalyzed by one enzyme (ColD), while two enzymes (E_1/E_3) are used in the formation of other 3,6-dideoxysugars. Second, in the ColD-catalyzed reaction, the cofactor (PMP) is regenerated by a second function of the enzyme, an aminotransferring activity, instead of its direct regeneration by the E_3-catalyzed reduction. The utilization of PMP in a dehydration is rare, but the combined deoxygenation-transamination activity makes ColD a unique enzyme. These findings are a strong testament to the phenomena that enzymes catalyzing the same type of reaction may exhibit glaring mechanistic differences, highlighting evolutionary diversity in biological deoxygenation events.[16]

Acknowledgment. This work is supported in part by the National Institutes of Health grant GM35906. T.M.H. was a trainee of a NIGMS Biotechnology Training Grant (2 T32 GM08347). H.-w.L. also thanks the National Institute of General Medical Sciences for a MERIT Award.

References

(1) (a) Whitfield, C. *Trends Microbiol*. **1995**, *3*, 178−185. (b) Saito, T.; Hatada, M.; Iwanaga, S.; Kawabata, S. *J. Biol. Chem*. **1997**, *272*, 30703−30708.

(2) (a) Weigel, T. M.; Liu, L.-d.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2129−2139. (b) Weigel, T. M.; Miller, V. P.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2140−2147. (c) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc*. **1993**, *115*, 7539−7540.

(3) (a) Miller, V. P.; Thorson, J. S.; Lo, S. F.; Ploux, O.; Liu, H.-w. *Biochemistry* **1993**, *32*, 11934−11942. (b) Lo, S. F.; Miller, V. P.; Lei, Y.; Thorson, J. S.; Liu, H.-w.; Schottel, J. L. *J. Bacteriol*. **1994**, *176*, 460−468.

(4) (a) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol*. **1994**, *48*, 223−256. (b) Hallis, T. M.; Liu, H.-w. *Acc. Chem. Res*. **1999**, *32*, 579−588. (c) He, X.; Agnihotri, G.; Liu, H.-w. *Chem. Rev*. **2000**, *100*, 4615−4661. (d) He, X.; Liu, H.-w. *Annu. Rev. Biochem*. **2002**, *71*, 701−754.

(5) Elbein, A. D.; Heath, E. C. *J. Biol. Chem*. **1965**, *240*, 1926−1931.

(6) The genes responsible for colitose biosynthesis in the *Escherichia coli 0111 O*-antigen have also been reported. The translated sequence of *colD* and that of its homologue in *E. coli*, *wbdK*, show excellent similarity (71% identity and 82% similarity) (Bastin, D. A.; Reeves, P. R. *Gene* **1995**, *164*, 17−23).

(7) Thorson, J. S.; Lo, S. F.; Ploux, O.; He, X.; Liu, H.-w. *J. Bacteriol*. **1994**, *176*, 5483−5493.

(8) An apparent M_r of 75.5 kDa estimated by gel filtration and a calculated mass of 44.5 kDa per monomer indicate that ColD exists as a homodimer in its native state.

(9) (a) Lei, Y.; Ploux, O.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4643−4654. (b) Pascarella, S.; Bossa, F. *Protein Sci*. **1994**, *3*, 701−705.

(10) A typical assay mixture contained 1.8 mM GDP-mannose, 40 μM PLP, 2 mM L-glutamate, 1.5 μM ColB, and 0.65 μM ColD in 200 μL of 50 mM potassium phosphate buffer (pH 7.0). The incubation was conducted at 37 °C for 30 min. The resulting mixture was separated by HPLC on an Econosil C18 column (4.6 × 250 mm) which was eluted isocratically with 2% acetonitrile in 50 mM triethylammonium acetate buffer (pH 6.8). The flow rate was 1 mL/min, and the detector was set at 254 nm. Retention times of the substrate and products were as follows: GDP-mannose (**2**), 5.1 min; GDP-4-keto-6-deoxy-mannose (**3**), 6.2 min; GDP-4-keto- 3,6-dideoxymannose (**4**), 8.8 min.

(11) (a) Evangelopoulos, A. E. *Chemical and Biological Aspects of Vitamin B_6 Catalysis*; A. R. Liss: New York, 1984. (b) Dolphin, D., Poulson, R., Avramovic, O., Eds. *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*; Wiley-Interscience: New York, 1986; Parts A and B. (c) John, R. A. *Biochim. Biophys. Acta* **1995**, *1248*, 81−96.

(12) A large scale incubation contained 47.5 mM GDP-mannose, 1.5 mM PLP, 32.4 mM L-glutamate, 0.13 mM ColB, 3.46 μM ColD in 250 μL of 50 mM sodium phosphate buffer (pH 7.0). The incubation was conducted at 37 °C overnight. The proteins were removed by filtration through a microcon YM-10 membrane, and the filtrate was purified by HPLC using an Econosil C18 column (10 × 250 mm) as described above in ref 10. The pooled fractions containing product **4** were desalted using two consecutive Econo-Pac 10 DG (Bio-rad) columns, and product elution was achieved using water. The purified **4**, after lyophilization, was stored at −80 °C.

(13) ^{1}H NMR (^2H_2O): δ 7.99 (1H, s), 5.82 (1H, d, J = 5.9 Hz), 5.39 (1H, bd, J = 7.5 Hz, 1-H), 4.50 (1H, q, J = 6.6 Hz, 5-H), 4.41 (1H, m), 4.24 (2H, m, including 2-H), 4.10 (2H, m), 2.93 (1H, dd, J = 15.5, 4.0 Hz, 3-H_{eq}), 2.34 (1H, dd, J = 15.5, 3.0 Hz, 3-H_{ax}), 1.14 (3H, d, J = 6.6 Hz, 5-Me). ^{13}C NMR (^2H_2O): δ 211.2 (C-4), 159.2, 154.2, 152.0, 137.9, 95.1 (d, J = 6.0 Hz, C-1), 87.1, 84.0 (d, J = 9.1 Hz), 73.8, 72.7 (C-5), 70.6, 70.1 (d, J = 10.1 Hz, C-2), 65.6 (d, J = 5.0 Hz), 42.4 (C-3), 13.8 (C-6). High-resolution negative ion FABMS calcd for $C_{16}H_{22}N_5O_{14}P_2$ $[M - H]^+$ 570.0639, found *m*/*z* 570.0636.

(14) The conversion was most effective at 37 °C, and the optimal pH was determined to be 7.0. Under such conditions, a K_m of 8.5 ± 0.1 μM for GDP-4-keto-6-deoxy-D-mannose **3** and a k_{cat} of 0.6 s^{-1} were determined. The corresponding K_m and k_{cat}/K_m values for L-glutamate are 1.4 ± 0.2 mM and 423 M^{-1} s^{-1}, respectively.

(15) (a) Miles, E. W. In *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1986; Part B, pp 253−310. (b) Hayashi, H. *J. Biochem*. **1995**, *118*, 463−473.

(16) 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; pp 351−396.

JA030088R