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COMMUNICATIONS

Published on Web 07/22/2006

The Bifunctional Glyceryl Transferase/Phosphatase OzmB Belonging to the HAD Superfamily That Diverts 1,3-Bisphosphoglycerate into Polyketide Biosynthesis

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FK520, tautomycin, lankamycin, concanamycin, geldanamycin, zwittermycin, maytansinoids, and oxazolomycin are polyketide natural products that have one or more glycolytic derived units in their backbone.[1,2] The biosynthetic gene clusters for FK520, tautomycin, oxazolomycin, concanamycin, geldanamycin, and the maytansinoid ansamitosin P-3 have been identified.[1] Each of these clusters contains a gene whose deduced product (a 40 kDa protein) belongs to the haloacid dehalogenase (HAD) superfamily (Figure S1) and is the most likely candidate for diverting a portion of the glycolytic pool into secondary metabolite polyketide biosynthesis.[1−3] In this paper, we show that OzmB, a member of the HAD family of proteins from the oxazolomycin biosynthetic pathway, is indeed diverting a portion of the glycolytic intermediate into the biosynthesis of polyketides, such as oxazolomycin and tautomycin. It is demonstrated that OzmB first sequesters D-1,3-bisphosphoglycerate from the glycolytic pool to form the D-3-phosphoglyceryl-S-OzmB intermediate, then removes the phosphate group to afford the D-3-glyceryl-S-OzmB species (acting as a phosphatase), and finally transfers the glyceryl group to an acyl carrier protein (ACP) to set the stage for polyketide biosynthesis (acting as a glyceryl transferase) (Figure 1). Each of the steps catalyzed by OzmB was monitored using nanospray Fourier transform ion cyclotron resonance mass spectrometry (nFT-ICRMS).

In primary metabolism, D-1,3-bisphosphoglycerate serves as a metabolic branch point as a precursor to serine, glyceryl lipids, lactate, and glucose 6-phosphate. Since D-1,3-bisphosphoglycerate is not commercially available, it was generated in situ using D-3-phosphoglycerate, ATP, and D-3-phosphoglycerate kinase in this study and utilized without further purification. Thus, incubation of OzmB with D-3-phosphoglycerate, ATP, Mg^{2+}, and D-3-phosphoglycerate kinase resulted in the addition of 88.8 Da due to loading of OzmB with glycerate (Figure 2B) and is, within experimental error, in agreement with the expected mass increase for glycerate (+88.02 Da). Removing ATP (Figure 2A) or D-3-phosphoglycerate abolished this activity, while removing D-3-phosphoglycerate kinase resulted in loading of a trace amount of glycerate due to background kinase activity (Figure S2), serving as negative controls. This means that, in order to divert D-1,3-bisphosphoglycerate into polyketide biosynthetic pathways, it is activated via covalent attachment to OzmB. In addition to the covalent tethering of D-3-phosphoglycerate, OzmB must also catalyze dephosphorylation at the 3-position to generate the glyceryl-S-OzmB species.

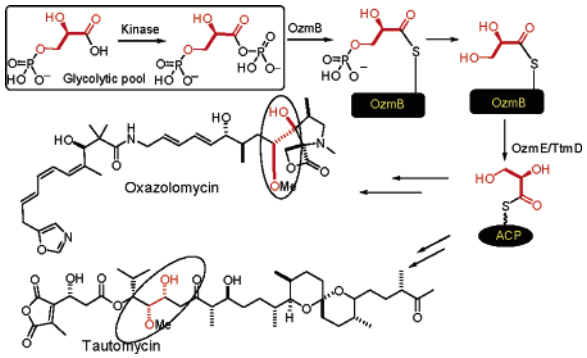

Figure 1. The diversion of D-1,3-bisphosphoglycerate into the biosynthesis of polyketides, such as oxazolomycin and tautomycin.

To map the attachment site of OzmB, the 51+ charge state of glyceryl-S-OzmB was first subjected to tandem mass spectrometry using either collisionally activated dissociation (CAD) or infrared multiphoton dissociation (IRMPD). This resulted in 7*b* and 13*y* unique fragment ions (Figure 2E).[4,5] This localized the glyceryl addition to the 142 amino acid stretch S_{215} to D_{357}. The *y*220 and *y*227 fragment ions, with mass values of 24802.2 and 25517.1 Da containing glycerate, were very abundant. Both of these ions are N-terminal to proline. Amides on the N-terminal side of proline are known to be preferentially fragmented during thermal activation and therefore often present themselves as the most abundant ions during CAD and IRMPD.[6] In an effort to further localize the position of glycerate on OzmB, these two abundant fragment ions were generated using CAD then isolated and subjected to IRMPD in the cell of the ICRMS instrument. This resulted in 24*y* and 17*b* fragment ions, further localizing the attachment site for glycerate to the 29 amino acids R_{252} to E_{283} (Figure 2E). Finally, glyceryl-S-OzmB was subjected to trypsin digestion and HPLC separation. The fraction eluting at 19−20 min contained the glyceryl-tethered form of the active site corresponding to G_{220} to R_{278} (M_r = 6472.64 Da). Subjecting this ion to IRMPD resulted in 16*b* and 14*y* fragment ions and localized the glycerate to the four residues $C_{266}RVV_{270}$, as all the *y*-fragments to the left (toward the N-terminal end) of the CRVV sequence correspond to a mass of the peptide +88.02 Da, while the *y*-fragment ions to the right (C-terminal end) of this sequence match the unmodified form of the peptide. Since the −SH group of cysteine is the only nucleophile in this four amino acid stretch, glycerate was assigned to be covalently attached to the side chain of the cysteine residue as a thioester.

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10.1021/ja0639362 CCC: $33.50

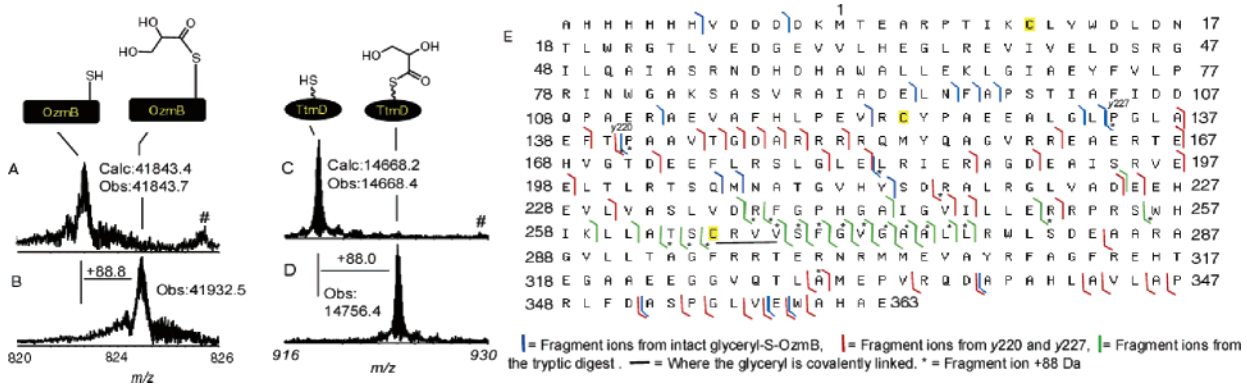

Figure 2. Loading and localization of glycerate on OzmB and its transfer to holo-TtmD ACP: (A) OzmB incubated with D-3-phosphoglycerate, D-3-phosphoglycerate kinase; (B) same as (A) plus ATP; (C) holo-TtmD; (D) holo-TtmD incubated with glyceryl-S-OzmB; and (E) localization of the covalent attachment site of glyceryl on OzmB. # = +178 Da covalent addition due to gluconylation of the N-terminal His_6-tag of OzmB or TtmD during overproduction in *E. coli*.[13]

To show that glyceryl-S-OzmB could transfer glyceryl to the −SH group of the 4′-phosphopantetheine moiety of ACP, it was co-incubated with the TtmD ACP from the tautomycin biosynthetic gene cluster (Figure S3).[7] To generate the holo-form of TtmD, it was incubated with CoA and the phosphopantetheinyl transferase Sfp.[8] Incubation of holo-TtmD with glyceryl-S-OzmB, generated using OzmB, ATP, D-3-phosphoglycerate, and D-3-phosphoglycerate kinase in situ, resulted in the addition of 88.0 Da onto holo-TtmD, consistent with the formation of the glyceryl-S-TtmD species. This addition was not observed when holo-OzmB, ATP, or D-3-phosphoglycerate was omitted (Figure 2C), but loading of a trace amount of glycerate was observed when the kinase was omitted, again due to background kinase activity (Figure S4), serving as negative controls.

After establishing the substrate specificity and mechanism of loading for OzmB, the phosphatase activity of OzmB and precise timing of this event were examined. OzmB and homologous proteins from other glycerate-derived polyketide pathways belong to the HAD superfamily of phosphatases, and this superfamily contains three motifs with high sequence homology. Alignments with OzmB clearly revealed motifs 1 and 2, including the nucleophilic aspartate deemed critical for dephosphorylation, were conserved (Figure S1).[9] The conserved aspartate, D14, in OzmB was mutated to valine, and OzmB loading activity was monitored. Incubation of OzmB(D14V) under the identical conditions resulted in addition of 168 Da, in agreement with D-3-phosphoglycerate loading onto OzmB and abolishment of phosphatase activity (Figure S5).

The high substrate specificity for D-1,3-bisphosphoglycerate, covalent tethering of D-3-phosphoglycerate to OzmB as a thioester, subsequent dephosphorylation, and transfer of the resultant glyceryl moiety from OzmB to an ACP represent a novel entryway of a glycolytic metabolite into polyketide biosynthetic pathways. In the polyketide biosynthetic pathways, a variety of modifications of the glycerate precursor are observed to generate a 2-hydroxyproprionyl-S-ACP, a pyruvoyl-S-ACP, a hydroxymalonyl-S-ACP, an aminomalonyl-S-ACP, or a methoxymalonyl-S-ACP.[1,2] In addition, an OzmB-like domain that includes the HAD motif and the conserved cysteine for glycerylation is also found on BryA (Figure S1). This domain, like OzmB, is likely involved in the activation of a glycerate unit found in bryostatin, making it a general strategy for incorporating glycerate into secondary metabolites.[10] Such units could be utilized as novel precursors in the efforts to engineer organisms to produce new bioactive natural products.[11,12]

Acknowledgment. This work was supported in part by NIH postdoctoral fellowships GM073323 (P.C.D.), CA1059845 (SVL), Chinese Ministry of Science & Technology grant 2003CB114205 (Z.D.), and NIH Grants CA113297 (B.S.), GM067725 (N.L.K.), and NIH Independent Scientist Award AI51689 (B.S.). We thank Dr. Werner F. Fleck, Hans Knoell Institute for Natural Product Research, Jena, Germany, for the oxazolomycin producing *Streptomyces albus* JA3454, and Dr. Hiroyuki Osada, RIKEN, Wako, Japan, for the tautomycin producing *S. spiroverticillatus*.

Supporting Information Available: Experimental details and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0639362