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1-Methylthio-D-xylulose 5-Phosphate Methylsulfurylase: A Novel Route to 1-Deoxy-D-xylulose 5-Phosphate in *Rhodospirillum rubrum*

Benjamin P. Warlick,[†,§] Bradley S. Evans,[§] Tobias J. Erb,[†,§,¶] Udipi A. Ramagopal,[⊥,△] Jaya Sriram,[∥] Heidi J. Imker,[§] J. Michael Sauder,[@] Jeffrey B. Bonanno,[⊥] Stephen K. Burley,[@] F. Robert Tabita,[∥] Steven C. Almo,[⊥] Jonathan S. Sweedler,[‡,§] and John A. Gerlt*[,†,‡,§]

[†]Department of Biochemistry and [‡]Department of Chemistry, 600 South Mathews Avenue, and [§]Institute for Genomic Biology, 1206 West Gregory Drive, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States

[∥]Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210, United States

[⊥]New York SGX Research Center for Structural Genomics, Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461, United States

[@]Lilly Biotechnology Center, 10300 Campus Point Drive, San Diego, California 92121, United States

Supporting Information

ABSTRACT: *Rhodospirillum rubrum* produces 5-methylthioadenosine (MTA) from *S*-adenosylmethionine in polyamine biosynthesis; however, *R. rubrum* lacks the classical methionine salvage pathway. Instead, MTA is converted to 5-methylthio-D-ribose 1-phosphate (MTR 1-P) and adenine; MTR 1-P is isomerized to 1-methylthio-D-xylulose 5-phosphate (MTXu 5-P) and reductively dethiomethylated to 1-deoxy-D-xylulose 5-phosphate (DXP), an intermediate in the nonmevalonate isoprenoid pathway [Erb, T. J., et al. (2012) *Nat. Chem. Biol.*, in press]. Dethiomethylation, a novel route to DXP, is catalyzed by MTXu 5-P methylsulfurylase. An active site Cys displaces the enolate of DXP from MTXu 5-P, generating a methyl disulfide intermediate.

Sequence-diverse RuBisCO-like proteins (RLPs), members of the D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) superfamily,[1,2] catalyze reactions in two different pathways by which the ribose moiety of 5-methylthioadenosine (MTA) generated in polyamine biosynthesis is recycled. In Bacilli, a RLP catalyzes enolization of 2,3-diketo-5-methylthiopentane 1-phosphate (DK-MTP 1-P) to 2-hydroxy-3-keto-5-thiomethylpent-1-ene 1-phosphate (HK-MTP 1-P) in the classical methionine salvage pathway (Figure 1A).[3] In *Rhodospirillum rubrum*, a distinct RLP (MTRu 1-P 1,3-isomerase) catalyzes isomerization of 5-methylthio-D-ribulose 1-phosphate (MTRu 1-P) to a 3:1 mixture of 1-methylthio-D-xylulose 5-phosphate (MTXu 5-P) and 1-methylthio-D-riblulose 5-phosphate [MTRu 5-P (Figure 1B)].[4] Genetic/metabolomic studies established that MTXu 5-P is the precursor to 1-deoxy-D-xylulose 5-phosphate (DXP) in the nonmevalonate pathway for isoprenoid synthesis, with the reductive loss of methanethiol catalyzed by a member of the cupin superfamily.[5] We now report structural and functional characterization of one such cupin.

In organisms that encode orthologs of MTRu 1-P 1,3-isomerase, a proximal gene encodes a member of the cupin superfamily. The cupin from *R. rubrum* was expressed in *Escherichia coli*, and its X-ray structure was determined at 2.3 Å resolution. Cys 121 is proximal to a Mn^{2+} ion (Figure 2) that can coordinate to the substrate and stabilize an enolate anion intermediate (vide infra).

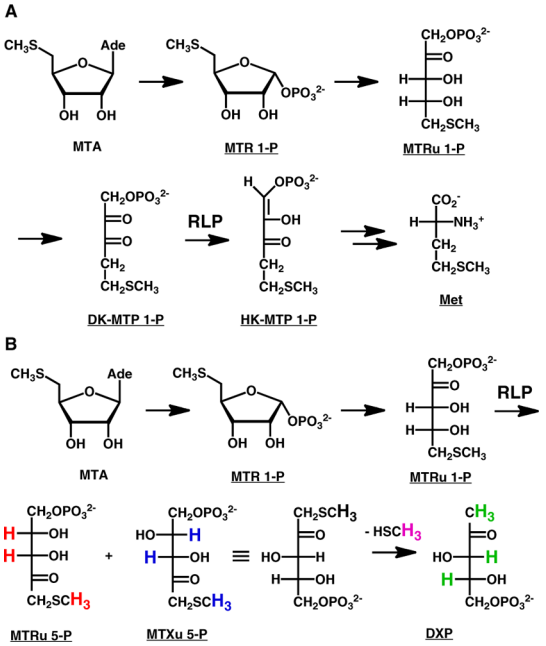

Figure 1. Reactions catalyzed by RLPs (A) in the classical methionine salvage pathway in Bacilli and (B) in the production of DXP in *R. rubrum*.

In initial studies using ^{1}H NMR, no reaction was observed with a mixture of MTRu 5-P and MTXu 5-P. However, when incubations using lower substrate concentrations were analyzed by mass spectrometry (MS), formation of DXP and methanethiol from MTXu 5-P was observed (two-electron

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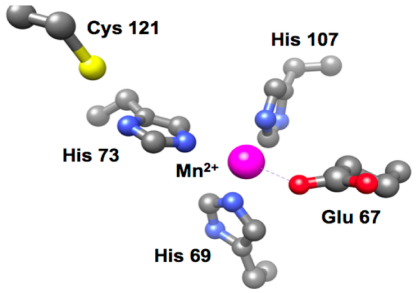

Figure 2. Active site of the cupin from *R. rubrum* (Protein Data Bank entry 3JZV).

reduction); the MTRu 5-P is inert and remains in the reaction mixture. Realizing that the cupin was stored in buffer containing DTT and that a molar excess of DTT was present in the incubations monitored by MS but not by NMR, we expected that DXP would be observed by ^{1}H NMR if a molar excess of DTT were present. As shown in Figure 3B, DXP and methanethiol were produced in the presence of excess DTT.

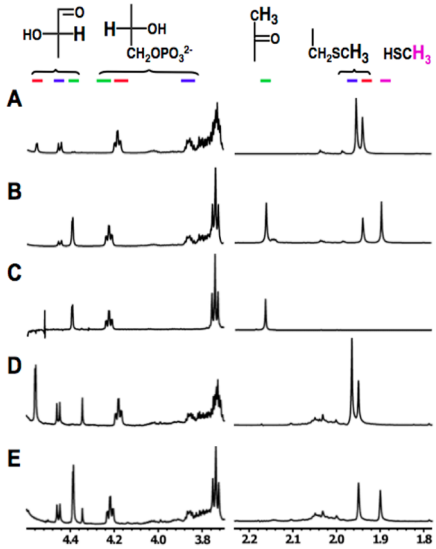

Figure 3. ^{1}H NMR spectra of the cupin substrates and products. (A) Mixture of MTRu 1-P and MTXu 1-P in H_2O. (B) After reaction with cupin in H_2O in the presence of excess DTT (spectrum obtained with solvent suppression). (C) Authentic DXP in H_2O. (D) Mixture of MTRu 1-P and MTXu 1-P in D_2O. (E) After reaction with cupin in D_2O. The resonances are color-coded according to the structures in Figure 1.

Two mechanisms can be envisioned for formation of DXP (Figure 4): (1) β-elimination of methanethiol followed by reduction of the 3,4-diketo intermediate (panel A) and (2) attack of Cys 121 to eliminate the DXP enolate anion to form a methyl disulfide adduct followed by reduction to release methanethiol (panel B). In both mechanisms, the enolate anion intermediate(s) can be stablilized by coordination to the metal ion (Figure 2).

To distinguish between these mechanisms, the cupin was incubated with a mixture of MTRu 5-P and MTXu 5-P in the absence of DTT (with "no" formation of DXP); the molecular weight of the cupin was determined by MS. Prior to incubation, the molecular weight was that predicted from the amino acid sequence; following incubation, the molecular weight increased

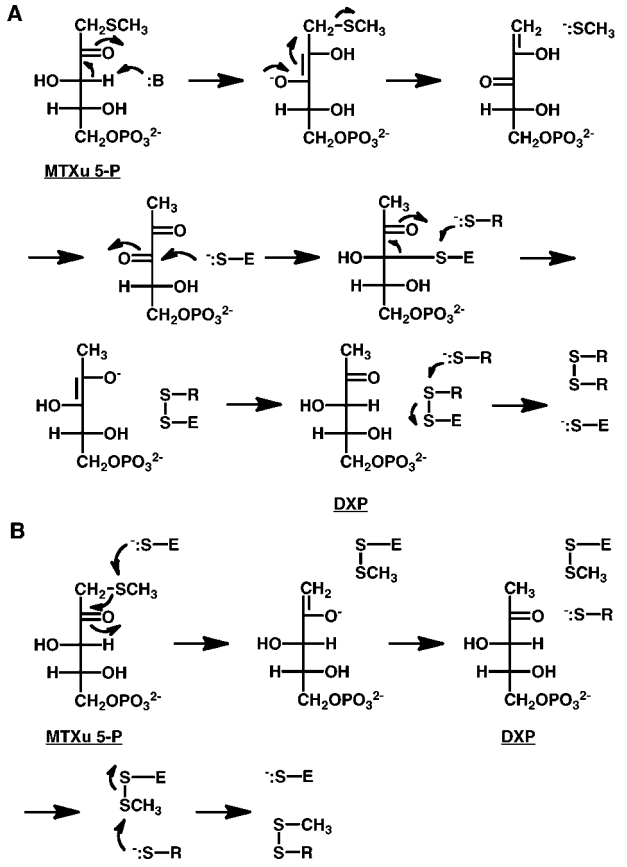

Figure 4. Possible mechanisms for the cupin-catalyzed reaction. (A) Proton abstraction followed by elimination and reduction. (B) Displacement of the enolate anion of DXP followed by reduction.

by 46 units, consistent with the incorporation of methanethiol (CH_3S). Trypsin digestion followed by tandem mass spectrometry of the covalently modified cupin localized the methanethiol to Cys 121 (Figure 5). This observation suggests that a disulfide is formed between Cys 121 and the methanethiol group of the substrate.

The cupin also was incubated with MTRu 5-P and MTXu 5-P in D_2O; the ^{1}H NMR spectrum (Figure 3E) revealed that solvent deuterium was incorporated into the methyl group of DXP but not the C3 hydrogen; i.e., methanethiol was not produced by β-elimination. (The methyl group is fully deuterated, because the 1-methylene group of MTXu 5-P undergoes rapid exchange with solvent hydrogen.[4]) The results of the MS and NMR experiments are consistent with Cys 121 displacing the enolate anion of DXP to generate a methyl disulfide intermediate (Figure 4B); consistent with this mechanism, the C121A mutant displays no catalytic activity.

We designate this enzyme MTXu 5-P methylsulfurylase. The proposed mechanism is similar to that established for tetrachlorohydroquinone dehalogenase (Scheme 1).[6] In these reactions, glutathione attacks the intermediate obtained by chloride elimination to form a glutathionylated adduct; the adduct is reduced by displacement of the dihydroquinone product (a mimic of an enolate anion) by a second molecule of glutathione, generating glutathione disulfide.

The kinetic constants were measured at saturating concentrations of several reductants in a coupled-enzyme assay using DXP reductoisomerase (Table 1). Neither DTT nor dihydrolipoate can be the in vivo cosubstrate. And, our

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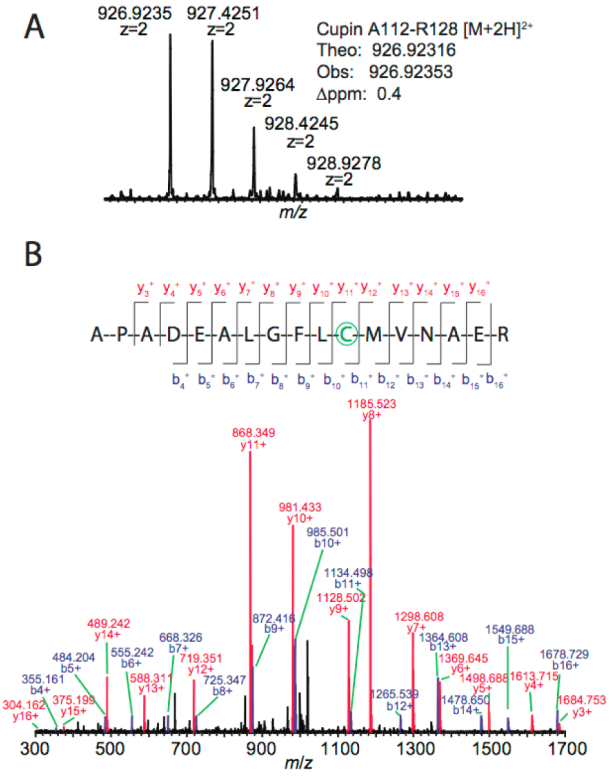

Figure 5. Localization of the methanethiol adduct to Cys 121. (A) Doubly charged tryptic peptide corresponding to residues 112−128 with a methanethiol disulfide modification. (B) Tandem mass spectrum of the peptide ion in panel A. The complementary fragment ion pair b_{10}^+ and y_{12}^+ localize the methanethiol modification to Cys 121.

Scheme 1

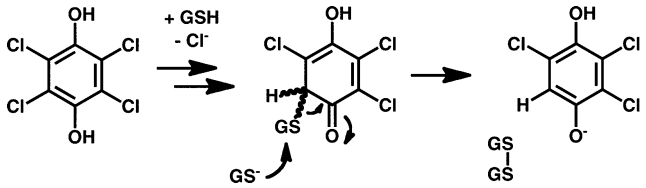

Table 1

reductant	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
DTT	7.8	39	2.0×10^5
coenzyme A	9.6	94	1.0×10^5
dihydrolipoate	4.3	46	9.3×10^4
Cys	2.1	42	5.1×10^4
glutathione	0.19	42	4.5×10^3

current metabolomics and transcriptomics data are insufficient to unequivocally identify the cosubstrate.[5] Regardless, we are confident that the conversion of MTXu 5-P to DXP is the physiological reaction.

The methylsulfurylase reaction allows the kinetic constants for the reaction catalyzed by MTRu 1-P 1,3-isomerase (the *R. rubrum* RLP) to be determined: k_{cat} = 1.3 s^{-1}, K_m = 50 μM, and $k_{cat}/K_m = 2.7 \times 10^4$ M^{-1} s^{-1}. In our initial report describing the 1,3-isomerase, we estimated the value of k_{cat} as 0.5 s^{-1} using ^{1}H NMR spectroscopy.[4] Together with our genetic and metabolomic evidence,[5] we now are confident that the conversion of MTRu 1-P to MTXu 5-P is the physiological reaction. We do not understand why MTRu 1-P 1,3-isomerase is promiscuous with respect to product formation (a 3:1 mixture of MTXu 5-P and MTRu 5-P): we have obtained no in vivo evidence that MTRu 5-P is the precursor to any metabolite. However, as expected, in vitro, the mixture of MTXu 5-P and MTRu 5-P is converted to DXP in the presence of the 1,3-isomerase.

With the functional characterization of the methylsulfurylase reported here, the pathway from MTA to DXP in *R. rubrum* (Figure 1B) is now established.

■ ASSOCIATED CONTENT

Supporting Information

A description of the experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (217) 244-7414. Fax: (217) 244-0508. E-mail: j-gerlt@illinois.edu.

Present Addresses

¶Institute of Microbiology, ETH Zurich, 8093 Zurich, Switzerland.

△Division of Biological Sciences, Poornaprajna Institute of Scientific Research, Bangalore − 560080, India.

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Notes

The authors declare no competing financial interest.

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