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Mechanistic Diversity in the RuBisCO Superfamily: RuBisCO from *Rhodospirillum rubrum* Is Not Promiscuous for Reactions Catalyzed by RuBisCO-like Proteins

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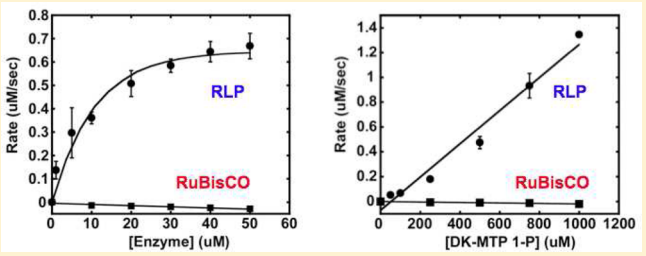
ABSTRACT: D-Ribulose 1,5-bisphosphate carboxylase/oxygenases (RuBisCOs) are promiscuous, catalyzing not only carboxylation and oxygenation of D-ribulose 1,5-bisphosphate but also other promiscuous, presumably nonphysiological, reactions initiated by abstraction of the 3-proton of D-ribulose 1,5-bisphosphate. Also, RuBisCO has homologues that do not catalyze carboxylation; these are designated RuBisCO-like proteins or RLPs. Members of the two families of RLPs catalyze reactions in the recycling of 5′-methylthioadenosine (MTA) generated by polyamine synthesis: (1) the 2,3-diketo-5-methylthiopentane 1-phosphate (DK-MTP 1-P) "enolase" reaction in the well-known "methionine salvage" pathway in *Bacillus* sp. and (2) the 5-methylthio-D-ribulose 1-phosphate (MTRu 1-P) 1,3-isomerase reaction in the recently discovered "MTA-isoprenoid shunt" that generates 1-deoxy-D-xylulose 5-phosphate for nonmevalonate isoprene synthesis in *Rhodospirillum rubrum*. We first studied the structure and reactivity of DK-MTP 1-P that was reported to decompose rapidly [Ashida, H., Saito, Y., Kojima, C., and Yokota, A. (2008) *Biosci., Biotechnol., Biochem. 72*, 959−967]. The 2-carbonyl group of DK-MTP 1-P is rapidly hydrated and can undergo enolization both nonenzymatically and enzymatically via the small amount of unhydrated material that is present. We then examined the ability of RuBisCO from *R. rubrum* to catalyze both of the RLP-catalyzed reactions. Contrary to a previous report [Ashida, H., Saito, Y., Kojima, C., Kobayashi, K., Ogasawara, N., and Yokota, A. (2003) *Science 302*, 286−290], we were unable to confirm that this RuBisCO catalyzes the DK-MTP 1-P "enolase" reaction either in vitro or in vivo. We also determined that this RuBisCO does not catalyze the MTRu 1-P 1,3-isomerase reaction in vitro. Thus, although RuBisCOs can be functionally promiscuous, RuBisCO from *R. rubrum* is not promiscuous for either of the known RLP-catalyzed reactions.



D-Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes carboxylation of D-ribulose 1,5-bisphosphate with atmospheric CO_2 to generate two molecules of 3-phosphoglycerate (3-PGA) in the Calvin−Benson−Bassham cycle as well as nonproductive oxygenation to generate one molecule of 3-PGA and one molecule of phosphoglycolate.[2] The reactions are initiated by abstraction of the substrate's 3-proton by a carboxylated active site Lys to generate an enediolate anion intermediate that is stabilized by coordination to an active site Mg^{2+} (Figure 1A). In addition, RuBisCOs catalyze other competing reactions in vitro that also are initiated by abstraction of the 3-proton (epimerization to generate D-xylulose 1,5-bisphosphate, 1,2-proton transfer to generate 3-ketoarabinitol 1,5-bisphosphate, and elimination of phosphate to generate 1-deoxy-D-*glycero*-2,3-pentdiulose 5-phosphate).[3−5]

The potential for RuBisCO to be promiscuous evidently has been exploited by Nature in the divergent evolution of homologues that do not catalyze the carboxylation of D-ribulose 1,5-bisphosphate, although the pathway(s) for the evolution of different physiological functions is not known. These homologues, designated RuBisCO-like proteins or RLPs, lack one or more functional groups necessary for carboxylation.[6,7] RLPs can be partitioned into several families (a sequence similarity network[8] for the RLPs is shown in Figure 2), some retaining cationic side chains that form the binding site for the 5-phosphate group of D-ribulose 1,5-bisphosphate in RuBisCO and others having hydrophobic substitutions for these residues that would prevent the binding of metabolites with 5-phosphate groups [all retain the binding site for the 1-phosphate group at the ends of the seventh and eighth β-strands in the $(\beta/\alpha)_8$-barrel domain].[9]

The members of two RLP families that lack the residues for binding a 5-phosphate group and, therefore, are predicted to utilize ketose 1-phosphates as substrates (instead of ketose 1,5-bisphosphates) participate in distinct pathways for recycling 5′-methylthioadenosine (MTA) generated in polyamine synthesis. The members of the first family (red nodes in Figure 2) catalyze the 2,3-diketo-5-methylthiopentane 1-phosphate (DK-MTP 1-P) "enolase"[a] reaction (Figure 1B) in the well-known "methionine salvage" pathway in *Bacillus* sp. in which

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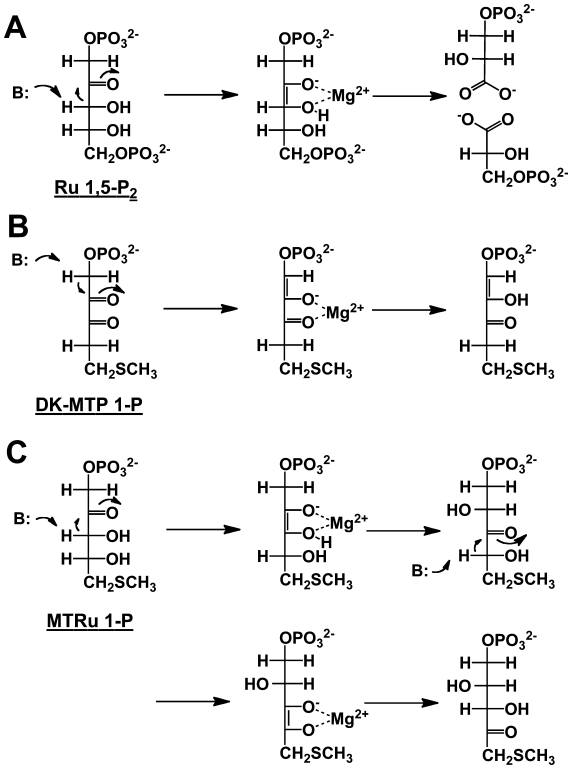

Figure 1. Mechanisms of RLP-catalzyed reactions: (A) RuBisCO, (B) DK-MTP 1-P "enolase", and (C) MTRu 1-P isomerase.

the 5′-methylthioribose moiety of MTA is converted to methionine (Figure 3A; in other organisms, the "enolase" reaction is catalyzed by an analogous enzyme); the product of the "enolase" reaction is 2-hydroxy-3-keto-5-methylthiopent-1-ene 1-phosphate (HK-MTP 1-P).[10] The members of the second family (blue nodes in Figure 2) catalyze the 5-methylthio-D-ribulose 1-phosphate (MTRu 1-P) 1,3-isomerase reaction (Figure 1) in a recently discovered nonmevalonate isoprene synthesis pathway ("MTA-isoprenoid shunt") in *Rhodospirillum rubrum* (Figure 3B);[11] the 1,3-isomerase converts MTRu 1-P to 1-methylthioxylulose 5-phosphate (MTXu 5-P) that is the immediate precursor to 1-deoxy-D-xylulose 5-phosphate (DXP) in the isoprene pathway and methanethiol that is captured, in part, for methionine synthesis.[12]

In the functional characterization of the RLP from *Bacillus subtilis* as the "enolase" in the methionine salvage pathway, data were provided that showed RuBisCO from *R. rubrum* could catalyze the "enolase" reaction.[10] A plasmid encoding RuBisCO from *R. rubrum* was reported to *trans*-complement a *B. subtilis* RLP$^-$ strain when MTA was provided as the sole sulfur source, although the growth rate was slower than that of the wild-type strain. Purified RuBisCO from *R. rubrum* also was reported to catalyze the "enolase" reaction, although in contrast to the reaction observed with the authentic "enolase" from *B. subtilis*, the reaction catalyzed by the RuBisCO showed a curious lag in the production of HK-MTP 1-P product.

Given our interest in the functions and mechanisms of the functionally characterized RLPs,[1,7,11,13] we decided to (re)-investigate the ability of RuBisCO from *R. rubrum* to catalyze both the "enolase" and MTRu 1-P 1,3-isomerase reactions. In particular, we were intrigued that the specificity-determining residues differ and, also, the proton abstraction reactions are catalyzed by structurally distinct general basic catalysts located on opposite faces of the 2-keto groups of the substrates that are coordinated to active site Mg^{2+} ions that stabilize the enediolate intermediates (the 3-proton of D-ribulose 1,5-bisphosphate by the carboxylated Lys in RuBisCO and the 1-pro-*S* proton of MTRu 1-P by a Lys that is present in all DK-MTP 1-P "enolases" but absent in RuBisCOs; we discuss the likely identities of the general basic and acidic catalysts in the 1,3-isomerase). In this article, we report that we are unable to reproduce the reported promiscuity of RuBisCO from *R. rubrum* to catalyze the "enolase" reaction in vitro or in vivo or detect the MTRu 1-P 1,3-isomerase reaction in vitro. We also comment on the limitations of using in vivo complementation without either detailed in vitro enzymology or a complete knowledge of in vivo metabolic pathways to discover functional promiscuity in the RuBisCO superfamily.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Polymerase chain reaction (PCR) amplification reactions were performed with Invitrogen Pfx polymerase according to the manufacturer's instructions; restriction digests were performed with enzymes obtained from New England Biolabs and used according to the manufacturer's instructions. ^{1}H NMR spectra were recorded with a Varian Unity INOVA 500NB spectrophotometer; ^{13}C NMR spectra were recorded with a Varian Unity Inova 600 MHz spectrometer. All kinetic assays were performed with a Cary 300 Bio UV−vis spectrometer.

5-Methylthio-D-ribose (MTR) was synthesized by published procedures.[1,11] RuBisCO from *R. rubrum*,[14] DK-MTP 1-P "enolase" from *Geobacillus kaustophilus*,[1] MTRu 1-P 1,3-isomerase from *R. rubrum*,[11] MTR 1-P 1,2-isomerase from *B. subtilis*,[1] and MTRu 1-P dehydratase from *B. subtilis*[1] were prepared according to procedures reported by our laboratories. $H_2{}^{18}O$ (95% enrichment) was purchased from Cambridge Isotope Laboratories, Inc.

[2-^{13}C]MTR. [2-^{13}C]MTR was prepared from [2-^{13}C]-D-ribose (Omicron Biochemicals) according to one of the procedures described for unlabeled MTR.[1]

MTR 1-P and [2-^{13}C]MTR 1-P. MTR 1-P and [2-^{13}C]MTR 1-P were prepared enzymatically using MTR kinase.[1]

^{1}H NMR Assay for RuBisCO. The reaction mixture (0.8 mL) contained 20 mM potassium phosphate (pH 7.8), 5 mM $MgCl_2$, 15 mM $NaHCO_3$, 2.5 mM D-ribulose 1,5-bisphosphate, and 0.25 μM RuBisCO. ^{1}H NMR spectra were recorded as a function of time. The intensity of the resonance associated with the 3-proton decreased linearly with time; the rate of this decrease was used to calculate the value of k_{cat}. The value of the ratio of carboxylation to oxygenation in the assay was calculated from the intensities of the resonances associated with the α-protons of 3-phosphoglyerate and 2-phosphoglycolate products.

Spectrophotometric Assay for Conversion of DK-MTP 1-P to HK-MTP 1-P ("Enolase" Reaction). The initial reaction mixtures (0.2 mL) contained 20 mM Na^+ HEPES (pH 7.9), 5 mM $MgCl_2$, 15 mM $NaHCO_3$, and MTR 1-P. MTRu 1-P was generated in situ by addition of MTR 1-P 1,2-isomerase to a final concentration of 1 μM followed by incubation at room temperature for 5 min. MTRu 1-P dehydratase then was added to a final concentration of 10 μM, and the reaction mixture was incubated for 5 min to allow formation of the hydrate of DK-MTP 1-P (vide infra).

To study the nonenzymatic conversion of DK-MTP 1-P to HK-MTP 1-P, the increase in absorbance at 270 nm was measured. To study the enzymatic conversion, either "enolase"

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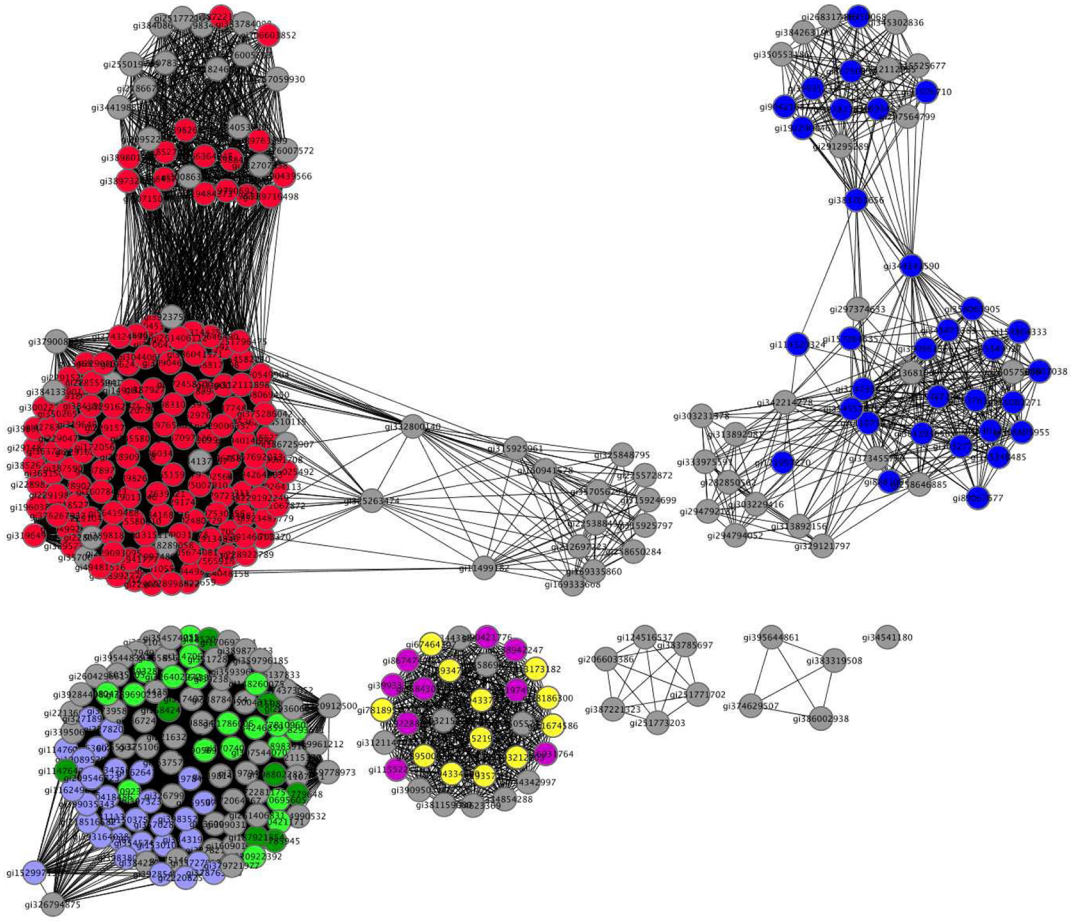

Figure 2. Sequence similarity network for the RLPs.[8] Each symbol (node) is a protein sequence; each line (edge) represents sequence similarity associated with the BLASTP *e* value relating the sequences. This network was constructed using 10^{-80} as the threshold *e* value; i.e., the edges are associated with values of $<10^{-80}$. The nodes colored red are associated with the DK-MTP 1-P "enolase" function; the nodes colored blue are associated with the MTRu 1-P 1,3-isomerase function. The functions associated with the other colors are currently unknown. The nodes colored light blue, light green, and green retain the residues for binding the ω-phosphate group of a ketose 1-phosphate substrate; the nodes colored magenta and yellow contain substitutions for these residues that suggest that the substrates for these are ketose 1-phosphates.

or RuBisCO was added, and the increase in absorbance at 270 nm was measured. The rate of formation of HK-MTP 1-P was calculated using an extinction coefficient of 9.1×10^3 M^{-1} cm^{-1} at 270 nm for the enone chromophore.[15]

For assays measuring the dependence of rate on enzyme concentration, the reaction mixtures contained 100 μM DK-MTP 1-P (from MTR 1-P). For assays measuring the dependence of the rate on DK-MTP 1-P concentration, "enolase" or RuBisCO was added to a final concentration of 1 or 10 μM, respectively.

Spectrophotometric Assay for Conversion of MTRu 1-P to MTXu 5-P (1,3-Isomerase Reaction). The initial reaction mixtures (0.2 mL) contained 100 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 15 mM $NaHCO_3$, 0.4 mM NADPH, 10 mM DTT, 10 μM D-xylulose 5-phosphate reductoisomerase, 1 μM 1-methylthio-D-xylulose 5-phosphate methylsulfurylase, and varying concentrations of MTR 1-P.[12] MTR 1-P 1,2-isomerase was added to a final concentration of 10 μM; after 5 min at room temperature, 1,3-isomerase or RuBisCO was added to a final concentration of 0.25 or 10 μM, respectively, and the reaction was monitored at 360 nm (ε = 4300 M^{-1} cm^{-1}).

^{1}H NMR for Conversion of DK-MTP 1-P to HK-MTP 1-P ("Enolase" Reaction). The initial reaction mixtures (0.8 mL) contained 20 mM potassium phosphate (pH 7.8), 5 mM $MgCl_2$, 15 mM $NaHCO_3$, and 5 mM MTR 1-P. (As specified in the figure legends, some reactions were performed in 100% D_2O; others were performed in a 90% H_2O/10% D_2O mixture.) MTR 1-P 1,2-isomerase was added to a final concentration of 5 μM, and the reaction mixture was incubated for 30 min to generate MTRu 1-P. For enzyme-catalyzed reactions, either "enolase" or RuBisCO was added to a final concentration of 1 μM before MTRu 1-P dehydratase was added to a final concentration of 1 μM was used to generate HK-MTP 1-P. For nonenzymatic reactions, only dehydratase was added. The reaction mixtures were incubated overnight at room temperature before the ^{1}H NMR spectra were recorded.

^{13}C NMR for Characterizing DK-MTP 1-P. For natural-abundance ^{13}C NMR spectra, the reaction mixtures (0.8 mL) contained 20 mM potassium phosphate (pH 7.8), 5 mM $MgCl_2$, and 10 mM MTR 1-P in a 90% H_2O/10% D_2O mixture. MTRu 1-P was formed in situ by the addition of MTR 1-P 1,2-isomerase to a final concentration of 5 μM. After 1 h, MTRu 1-P dehydratase was added to a final concentration of 1 μM, and the reaction mixture was immediately cooled to 4 °C in the spectrometer to suppress the rate of enolization to HK-MTP 1-P. A total of 22576 transients were acquired over 18 h.

^{13}C NMR Spectra of [2-^{13}C]MTR 1-P, [2-^{13}C]MTRu 1-P, and [2-^{13}C]DK-MTP 1-P. For the spectrum of [2-^{13}C]MTR 1-P, the reaction mixture (0.8 mL) contained 20 mM potassium

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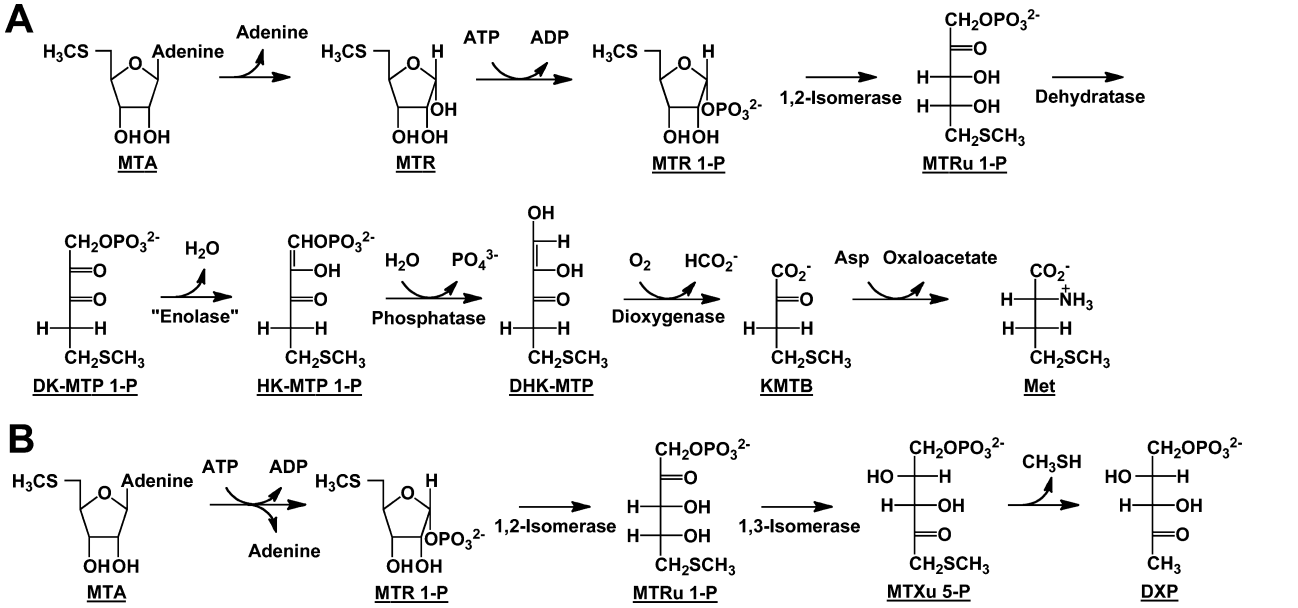

Figure 3. Two characterized pathways for MTA recycling. (A) Classical methionine salvage pathway in which methionine is quantitatively recovered from MTA.[10] (B) Recently discovered pathway in which MTA is the ribose moiety of MTA that is converted to DXP and the 5′-methylthio group is released as methanethiol that can be captured, in part, to form methionine.[12]

phosphate (pH 7.8), 5 mM $MgCl_2$, and 5 mM [2-^{13}C]MTR 1-P in a 50% $H_2{}^{18}O$/40% $H_2{}^{16}O$/10% $D_2{}^{16}O$ mixture. MTR 1-P 1,2-isomerase then was added to a final concentration of 10 μM; after 1 h, the ^{13}C NMR spectrum of [2-^{13}C]MTRu 1-P was recorded. MTRu 1-P dehydratase then was added to a final concentration of 4 μM; after 1 h, the ^{13}C NMR spectrum of [2-^{13}C]DK-MTP 1-P was recorded.

^{1}H NMR for Conversion of MTRu 1-P to MTXu 5-P/MTRu 5-P (1,3-Isomerase Reaction). The reaction mixtures (0.8 mL) contained 20 mM potassium phosphate (pH 7.8), 5 mM $MgCl_2$, 15 mM $NaHCO_3$, and 5 mM MTR 1-P in 100% D_2O. MTRu 1-P was formed in situ by addition of MTR 1-P 1,2-isomerase to a final concentration of 5 μM. After 30 min, either 1,3-isomerase or RuBisCO was added to a final concentration of 10 μM. The ^{1}H NMR spectrum was recorded after overnight incubation at room temperature.

Insertional Disruption of the Gene Encoding DK-MTP 1-P "Enolase" in *B. subtilis* (MtnW::pMUTIN4). The gene encoding the "enolase" (*mtn*W) in *B. subtilis* was inactivated by insertional disruption using the erythromycin resistance conferring plasmid pMUTIN4[16] according to the published procedure.[10] An ~350 bp segment of the N-terminal section of mtnW was amplified by PCR with primers containing a 5′ BamHI site and a 3′ HindIII site (5′-GGAGGATCCTACGCCAAACTTCGGTCCCGG-3′ and 5′-GGCATGAAGCTTTTATTAGCGACATATCTCCTGACCG-3′, respectively). The product was digested and ligated into disruption vector pMUTIN4.

Wild-type *B. subtilis* 168 was transformed with the pMUTIN4 construct[17] and spread on LB plates containing 1 μg/mL erythromycin, 1 mM IPTG, and 1 mM L-methionine. Colonies were allowed to grow at 37 °C for 48 h before they were screened. Extracted genomic DNA was screened by PCR amplification of the insert junctions using the following primers: 5′-GGCTGCCTGTGCCTGAAGGCTATACTTCTGAG-3′ and 5′-GGATGTGCTGCAAGGCGATTAAGTTGGGTAACG-3′ for the 5′-junction site and 5′-GGAGCTAAAGAGGTCCCTAGACTCTAGACCCGG-3′ and 5′-GCCATAATCGGAACTGGGATTTCAGGATCTTCTG-3′ for the 3′-junction site. The strain, designated MtnW::pMUTIN4, was unable to utilize MTA as the sole sulfur source in minimal medium.

Complementation with pDR67 MtnXYZ by Insertion into *amy*E. For addressing polar effects due to the insertional disruption of MtnW, the downstream genes encoding the phosphatase (MtnX), dehydratase (MtnY), and dioxygenase (MtnZ) were amplified[18] using the following primers: 5′-GGTCTAGACGCTAGATAAATGGGGAAAGG-3′ that introduces an XbaI cut site and 5′-GACAGCGTGAATCAATAAGCGAGATCTGCG-3′ that introduces a BglII cut site. After double digestion, the insert was ligated into pDR67 and screened for the presence of the insert. Prior to transformation, the pDR67 MtnXYZ plasmid was linearized with AatII overnight at 37 °C. The transformation of *B. subtilis* 168 (wild type and MtnW::pMUTIN4) with linearized pDR67 MtnXYZ was performed as described above.

Complementation with pEB112, pEB112 Containing *B. subtilis* RLP, and pEB112 Containing *R. rubrum* RuBisCO. The gene encoding MtnW was amplified via PCR using the following primers: 5′-TTTTCGGATCCTCATACGGCTTCAGCCTTTCCCCATTTATCTAGCG-3′ and 5′-GCGACCCGGGTTAAGGAGGATTTTTTGATATGGATGAAAATGAAAGG-3′ that introduce BamHI and XmaI restriction sites, respectively. The gene encoding *R. rubrum* RuBisCO was amplified via PCR from gDNA using the following primers: 5′-CGAGGATCCTTAAGGAGGATTTTTTGATATGGACAGTCATCTCGTTACGTCAATCTGG-3′ and 5′-GACGCGGAGGGGAGGGATCCCGCGTCTTCTGCTTTGACCGGCCGTCCAACC-3′ that introduce BamHI restriction sites. Both fragments were digested according to the manufacturer's specifications. Digested fragments were gel extracted using a Qiaquick Gel extraction kit and then ligated into predigested pEB112. Ligation mixtures were used to transform *Escherichia coli* XL-1 blue competent cells by electroporation. Colonies were screened for insert and correct orientation before they were used to transform *B. subtilis* 168 wild type or *B. subtilis* 168 MtnW::pMUTIN4. Transformations were plated on LB agar plates supplemented with 50 μg/mL kanamycin.

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Media and Conditions Used for Growth Curves. The measurement of growth curves used the ED minimal medium described previously[19] with the addition of 1 mM IPTG. When necessary, antibiotics were added: 1 μg/mL erythromycin, 1 μg/mL kanamycin, and/or 1 μg/mL chloramphenicol.

RESULTS AND DISCUSSION

Structure and Chemical Reactivity of DK-MTP 1-P. Knowledge of the solution structure and chemical properties of DK-MTP 1-P, the substrate for "enolase", is essential for understanding both its nonenzymatic and its enzymatic reactivity as investigated in this work. DK-MTP 1-P is generated in situ from MTRu 1-P by the action of MTRu 1-P dehydratase (Figure 3A). In the published characterization of dehydratase,[19] the DK-MTP 1-P product was reported to be labile, "decomposing" with a rate constant of 0.048 s^{-1} ($t_{1/2}$ ~ 15 s). The decomposition of DK-MTP 1-P was quantitated by the loss of absorbance at 270 nm (the α,β-diketo chromophore in DK-MTP 1-P) after its rapid in situ generation from MTRu 1-P. When dehydratase and "enolase" were present together in a reaction mixture containing MTRu 1-P, the rate of formation of the enolized HK-MTP 1-P product of "enolase" was significant; however, when "enolase" was added several minutes after dehydratase [~6 min or 24 half-times for decomposition of DK-MTP 1-P (Figure 4 of ref 19)], the rate of formation of HK-MTP 1-P was significantly decreased. When additional MTRu 1-P was added to the reaction mixture, the rate of formation of HK-MTP 1-P again was significant (as expected because the dehydratase and "enolase" were both present).

We used ^{1}H and ^{13}C NMR spectroscopies to characterize the structure of the "decomposed" DK-MTP 1-P product of dehydratase. The ^{1}H NMR spectrum (in a 90% H_2O/10% D_2O mixture) of a solution containing DK-MTP 1-P obtained within 2 min of generation with dehydratase is shown in Figure 4A (eight half-times for decomposition, therefore "decomposed"). The spectrum appears to be that expected for the structure of DK-MTP 1-P: a doublet associated with the protons on carbon-1 (3.8 ppm; coupled to ^{31}P of the 1-phosphate group), two triplets associated with the protons on carbon-4 and -5 (3.05 and 2.65 ppm, respectively), and a singlet associated with the thiomethyl group (2.0 ppm).

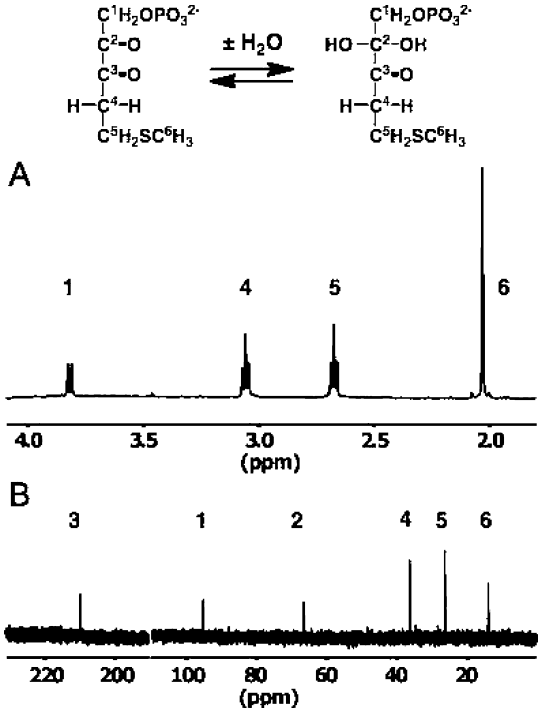

Figure 4. Solution characterization of DK-MTP 1-P. (A) ^{1}H NMR spectrum immediately after in situ generation from MTRu 1-P with MTRu 1-P dehydratase. (B) ^{1}H-decoupled natural-abundance ^{13}C NMR spectrum.

The natural-abundance ^{13}C NMR spectrum (Figure 4B) contained six resonances. However, only a single resonance was observed in the downfield region for carbonyl carbons (210 ppm); also, one resonance was observed in the region expected for a carbon with two singly bonded oxygens (95 ppm) and only a single resonance for a carbon with one singly bonded oxygen (68 ppm). However, as expected, the spectrum contained a resonance for a carbon adjacent to a carbonyl carbon (38 ppm) and two for carbons adjacent to sulfur (26 and 15 ppm). From this spectrum, we concluded that one of the α,β-diketo carbonyl carbons is hydrated (gem-diol) in "decomposed" DK-MTP 1-P. A straightforward interpretation of the lack of enzymatic reactivity would be that the 2-carbonyl group is hydrated, thereby decreasing the acidity of the 1-proton that would be abstracted in the "enolase" reaction. Alternatively, hydration of the 3-carbonyl group might prevent its binding to the Mg^{2+} that stabilizes the enolate anion intermediate in the active site of "enolase".

Verification that the 2-carbonyl group is, in fact, hydrated in the "decomposed" DK-MTP 1-P was established using [2-^{13}C]MTR 1-P as the precursor and performing successive in situ generation of [2-^{13}C]MTRu 1-P with MTR 1-P 1,2-isomerase and [2-^{13}C]DK-MTP 1-P with dehydratase in a 50% $H_2{}^{18}O$/40% $H_2{}^{16}O$/10% $D_2{}^{16}O$ mixture. The ^{1}H-decoupled spectrum of [2-^{13}C]MTR 1-P is a doublet because of coupling of the 2-^{13}C to the ^{31}P of the 1-phosphate group (Figure 5A). After isomerization, the ^{1}H-decoupled spectrum of [2-^{13}C]MTRu 1-P appears as a doublet of doublets (Figure 5B): (1) the 2-^{13}C is coupled to the ^{31}P of the 1-phosphate group, and (2) the carbonyl oxygen is in equilibrium with the 1:1 ^{16}O:^{18}O isotopic composition of the solvent (with the ^{18}O causing an upfield shift in the ^{13}C resonance). After dehydration, the ^{1}H-decoupled spectrum appears as a doublet of triplets (Figure 5C), establishing that the 2-carbonyl group is hydrated (present as a gem-diol): (1) the 2-^{13}C is coupled to the ^{31}P of the 1-phosphate group, and (2) both oxygens of the hydrate are equilibrated with the solvent, with the "triplet" reflecting the expected 1:2:1 isotopic distribution of the $^{16}O_2$-, $^{16}O^{18}O$-, and $^{18}O_2$-labeled hydrates.

When "decomposed", actually hydrated, DK-MTP 1-P is incubated in the absence of "enolase", the absorbance at 270 nm (also the enone chromophore in HK-MTP 1-P) slowly increases (Figure 6A) and the ^{1}H NMR spectrum reveals the formation of the enolized HK-MTP 1-P as judged by the characteristic vinyl resonance [a doublet because of coupling to the ^{31}P of the 1-phosphate group (Figure 6B)]. The rate constant describing the initial formation of HK-MTP 1-P, 0.0004 s^{-1} ($t_{1/2}$ ~ 30 min), is independent of substrate concentration; i.e., the enolization is first-order in DK-MTP 1-P. As shown in Figure 6A, the enolization is biphasic, "quickly" approaching ~60% completion in the time predicted by the measured rate constant; this phase is followed by a slower increase in absorbance until the reaction reaches equilibrium (~4:1 HK-MTP 1-P:DK-MTP 1-P ratio). No change is observed in the chemical shifts of the resonances in the ^{1}H NMR spectrum during the second phase, nor does a second set of resonances that can be associated with a different structure for

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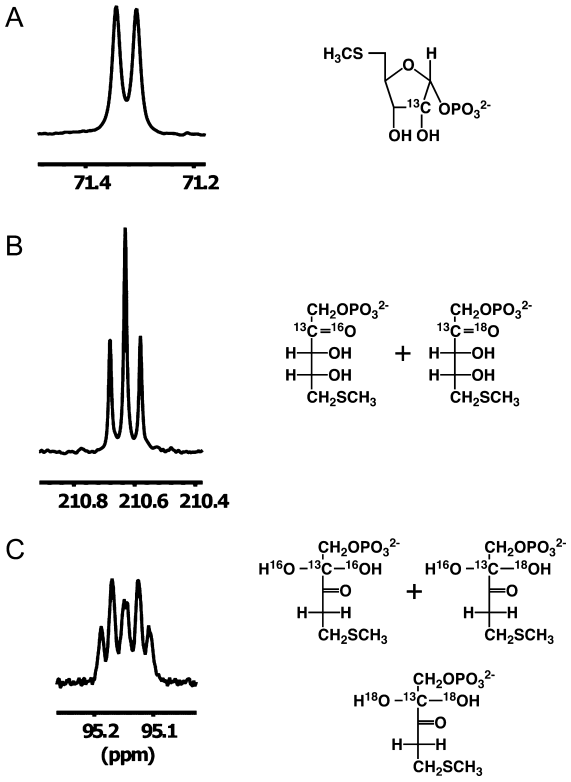

Figure 5. ^{13}C NMR spectra of 2-^{13}C-labeled materials in a 50% $H_2{}^{18}O$/40% $H_2{}^{16}O$/10% $D_2{}^{16}O$ mixture (i.e., the isotopic composition of the solvent is 50% ^{16}O and 50% ^{18}O): (A) [2-^{13}C]MTR 1-P, (B) [2-^{13}C]MTRu 1-P, and (C) [2-^{13}C]DK-MTP 1-P.

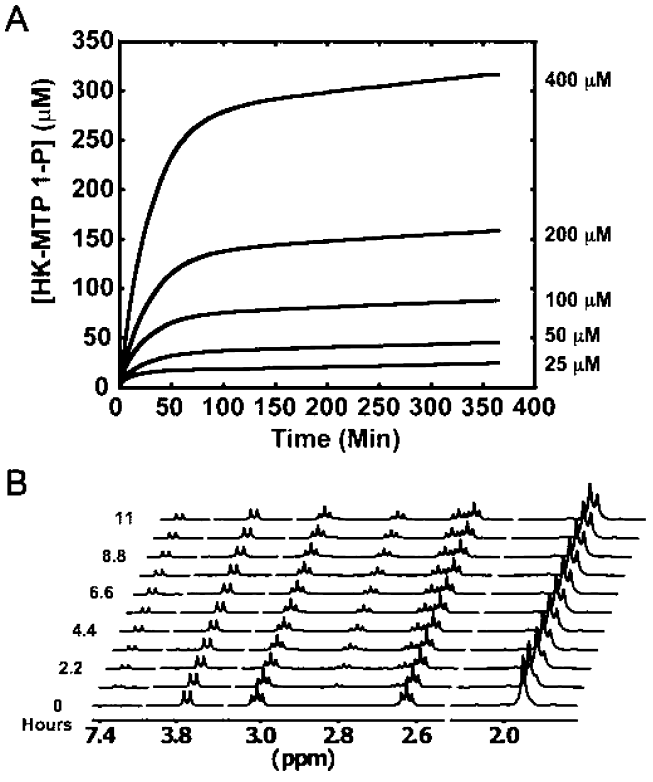

Figure 6. Nonenzymatic enolization of DK-MTP 1-P to HK-MTP 1-P. (A) Time course of the reaction at several DK-MTP concentrations monitored at 270 nm. (B) Time course of the reaction (5 mM DK-MTP 1-P) monitored by ^1H NMR spectroscopy. The assignments of the resonances are provided in Figure 4A.

HK-MTP 1-P appear. One explanation is that HK-MTP 1-P exists as a mixture of geometric isomers, i.e., *cis*- and *trans*-enediols, with one of these favored kinetically. Irrespective of the explanation, the hydrate of DK-MTP 1-P is not "decomposed" irreversibly but is capable of nonenzymatic enolization via the small amount of unhydrated DK-MTP 1-P present in equilibrium with the hydrate.

In Vitro Reactivity of DK-MTP 1-P with "Enolase" and RuBisCO ("Enolase" Reaction). Having characterized the structure of DK-MTP 1-P and the rate of its nonenzymatic "enolization", we studied the ability of both "enolase" and RuBisCO to catalyze the DK-MTP 1-P "enolase" reaction. The dependence of the reaction rate on enzyme concentration in shown in Figure 7A; the dependence of reaction rate on substrate concentration is shown in Figure 7B. (The k_{cat} for RuBisCO under these conditions measured by ^1H NMR spectroscopy is 0.85 s^{-1}; the ratio of carboxylation to oxygenation is ~1.)

"Enolase" catalyzes the slow, but easily measurable, formation of HK-MTP 1-P (k_{cat}/K_m = 1300 M^{-1} s^{-1}); the low value of k_{cat}/K_m is explained by our demonstration (previous section) that the reactive, unhydrated DK-MTP 1-P constitutes only a minor fraction of the total "substrate" pool (even with [2-^{13}C]DK-MTP 1-P,[3] no resonance associated with the 2-keto group can be detected). The value of k_{cat}/K_m for "enolase" is 1.3×10^7 M^{-1} s^{-1} when the reaction is initiated by the rapid formation of DK-MTP 1-P by addition of dehydratase to an assay mixture containing MTRu 1-P; i.e., the initial rate is measured before a significant amount of the hydrate is formed.[20] The ratio of the value of k_{cat}/K_m we measure after the hydrate is formed to the value before the hydrate is formed measures the fraction of DK-MTP 1-P that exists as the reactive diketo form, i.e., 0.01%.

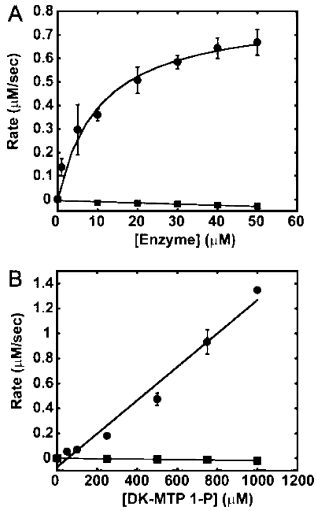

Figure 7. Reactivity of DK-MTP with DK-MTP 1-P "enolase" and RuBisCO. (A) Dependence of reaction velocity on enzyme concentration using 100 μM DK-MTP 1-P: (●) "enolase" and (■) RuBisCO. (B) Dependence of reaction velocity on substrate concentration: (●) 1 μM "enolase" and (■) 10 μM RuBisCO.

We attribute the "saturation" in rate as the enzyme concentration is increased to rate-limiting dehydration of the hydrate to the reactive unhydrated molecule. In contrast, even at high enzyme or substrate concentrations, no reaction is observed with RuBisCO and the in situ-produced DK-MTP 1-P

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($k_{cat}/K_m = -1.9 \pm 0.3\ M^{-1}\ s^{-1}$). Thus, we conclude that in vitro RuBisCO is not promiscuous for the "enolase" reaction.

We note that the genome of *R. rubrum* does not encode the classical methionine salvage pathway that includes the DK-MTP 1-P "enolase" (Figure 3A); instead, it converts MTA to DXP and methanethiol via the MTA-isoprenoid shunt, a newly discovered pathway, that includes the MTXu 5-P 1,3-isomerase reaction (Figure 3B) that also is catalyzed by an RLP.[11] Thus, even if it had been observed, no functional significance would have been easily attributed to a promiscuous DK-MTP 1-P "enolase" reaction catalyzed by RuBisCO. Finally, the lack of promiscuity of RuBisCO for the "enolase" reaction is expected because the single general basic catalysts in RuBisCO and the "enolase" active sites are located on opposite faces of their substituted D-ribulose substrates (vide infra).

In Vivo Reactivity of DK-MTP 1-P with RuBisCO. As noted previously, RuBisCO was reported to catalyze the "enolase" reaction in a *B. subtilis* strain in which the gene encoding the "enolase" (MtnW) had been insertionally disrupted (RLP^- strain) as judged by its ability to restore growth on MTA as the sole sulfur source.[10] This strain was constructed to have the downstream genes in the same operon encoding the dehydratase, phosphatase, and dioxygenase (Figure 3A, MtnXYZ) under the control of a separate promoter to eliminate polar effects. Because we observed that the RuBisCO was not able to catalyze the "enolase" reaction in vitro (vide supra), we repeated these complementation experiments.

In our experiments, the gene encoding the "enolase" was insertionally disrupted using pMUTIN4; i.e., we generated an independent RLP^- strain, and as expected, this strain (MtnW::pMUTIN4) was unable to utilize MTA as the sole sulfur source. Irrespective of whether it was transformed with a plasmid (pEB112) containing no insert ("empty" vector), the gene encoding "enolase", or the gene encoding RuBisCO, no complementation was observed, i.e., no growth on MTA as the sole sulfur source (data not shown). We attributed the lack of complementation by "enolase" to polar effects: as noted previously, the gene encoding "enolase" is upstream of those encoding dehydratase, phosphate, and dioxygenase, so its disruption likely decreases the efficiency of transcription of the downstream genes.

To circumvent polar effects, we inserted the genes encoding dehydratase, phosphate, and dioxygenase (MtnXYZ) into the *amy*E locus of our RLP^- strain (MtnW::pMUTIN4) used to generate the strain designated as MtnW::pMUTIN4 amyE::pDR67. With this strain, pEB112 containing the gene for "enolase" complemented the disruption, but pEB112 without an insert or containing the gene for RuBisCO did not restore the ability to use MTA as the sole sulfur source (Figure 8). [The RLP^- strain transformed with the empty vector is able to grow slowly with MTA as the sole sulfur source, likely because the DK-MTP 1-P produced the residual dehydratase nonenzymatically "enolizes" slowly in the absence of the "enolase" (insertionally disrupted so it is not present) and can be used as a substrate by the low levels of phosphatase and dioxygenase that also are present. We cannot provide an explanation for why the RLP^- strain transformed with the vector containing the RuBisCO gene grew more slowly than the strain transformed with the empty vector.]

Thus, we conclude from our in vitro and in vivo experiments that RuBisCO is unable to catalyze the "enolase" reaction. Although we cannot provide an explanation for why the results of our in vivo experiments differed from those reported previously,[10] we are confident that in vitro RuBisCO cannot catalyze the "enolase" reaction based on our thorough characterization of both the solution structure of DK-MTP 1-P and its reactivity for the formation of HK-MTP 1-P in the absence and presence of "enolase".

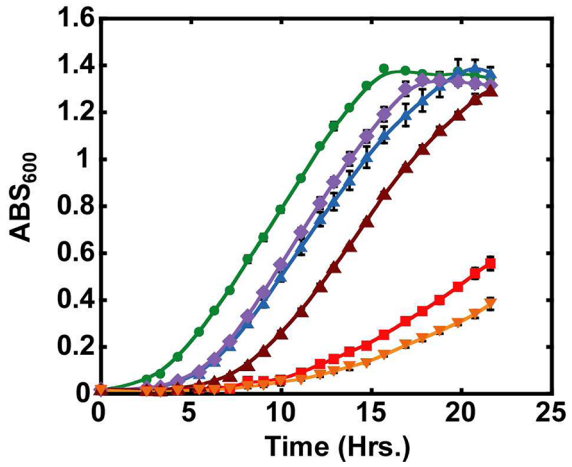

Figure 8. Growth curves of wild-type *B. subtilis* and *B. subtilis* with the "enolase" insertional deletion (MtnW::pMUTIN4). The data are for wild-type *B. subtilis* with the genes encoding the dehydratase, phosphatase, and dioxygenase inserted in *amy*E (amyE::pDR67 MtnXYZ) and transformed with empty pEB112 (green circles), wild-type amyE::pDR67 transformed with pEB112 containing the "enolase" (purple diamonds), wild-type amyE::pDR67 MtnXYZ transformed with pEB112 containing the RuBisCO form *R. rubrum* (blue triangles), *B. subtilis* with the insertional disruption of the "enolase" (MtnW::pMUTIN4) and amyE::pDR67 MtnXYZ transformed with empty pEB112 (red-orange squares), MtnW::pMUTIN4 and amyE::pDR67 MtnXYZ transformed with pEB112 containing the "enolase" (brown triangles), and MtnW::pMUTIN4 and amyE::pDR67 MtnXYZ transformed with pEB112 containing the RuBisCO form *R. rubrum* (orange inverted triangles).

Reactivity of MTRu 1-P with RuBisCO (1,3-Isomerase Reaction). Finally, we investigated whether RuBisCO is promiscuous for the reaction catalyzed by the RLP encoded by its genome, i.e., the 1,3-isomerase reaction (Figure 3B). We first used ^1H NMR spectroscopy to determine whether RuBisCO catalyzes the 1,3-isomerase reaction. After incubation of MTRu 1-P (Figure 9A) with 1,3-isomerase in D_2O, the previously documented 1:3 mixture of MTXu 5-P and MTRu 5-P was formed (Figure 9B[13]). (Note that the isomerase-catalyzed reaction is accompanied by incorporation of solvent hydrogen at carbon-1, -4, and -5.) However, no reaction was observed in the presence of RuBisCO (Figure 9C).

We also used a coupled-enzyme spectrophotometric assay to quantitate the activity of RuBisCO for the 1,3-isomerase reaction;[12] the formation of MTXu 5-P from MTRu 1-P is detected by its further conversion to DXP by DXP reductoisomerase.[12] With this assay, virtually no activity could be detected: $k_{cat}/K_m = 0.1 \pm 0.9\ M^{-1}\ s^{-1}$ for RuBisCO; $k_{cat} = 1.3\ s^{-1}$, $K_m = 50\ \mu M$, and $k_{cat}/K_m = 2.7 \times 10^4\ M^{-1}\ s^{-1}$ for the 1,3-isomerase reaction. Thus, we conclude that in vitro RuBisCO is not promiscuous for the 1,3-isomerase reaction.

We previously demonstrated that a mutant strain of *R. rubrum* in which the genes encoding both RuBisCO and 1,3-isomerase had been disrupted could not utilize MTA aerobically. Furthermore, although the gene encoding 1,3-isomerase could complement the aerobic growth phenotype, the gene encoding RuBisCO could not.[21] Thus, in vivo RuBisCO is not promiscuous for the 1,3-isomerase reaction.

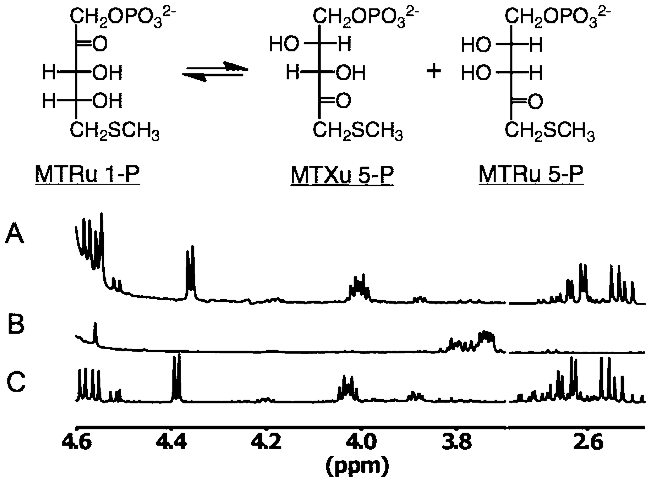

Figure 9. Reactivity of MTRu 1-P. (A) ^{1}H NMR spectrum of MTRu 1-P. (B) ^{1}H NMR spectrum of MTRu 1-P after overnight incubation with 1 μM MTRu 1-P 1,3-isomerase. (C) ^{1}H NMR spectrum of MTRu 1-P after overnight incubation with 10 μM RuBisCO.

An unliganded structure [Protein Data Bank (PDB) entry 3QFW] is available for a presumed orthologous 1,3-isomerase from *Rhodopseudomonas palustris*. The active sites of activated (carboxylated) RuBisCO from *R. rubrum* complexed with D-ribulose 1,5-bisphosphate (D-Ru 1,5-bisP, colored cyan in panel A, PDB entry 9RUB), activated (carboxylated) "enolase" from *G. kaustophilus* complexed with 2-hydroxy-3-keto-5-hydroxypentane 1-phosphate (DK-H 1-P, colored magenta, PDB entry 2OEM), and 1,3-isomerase are compared in Figure 10. In

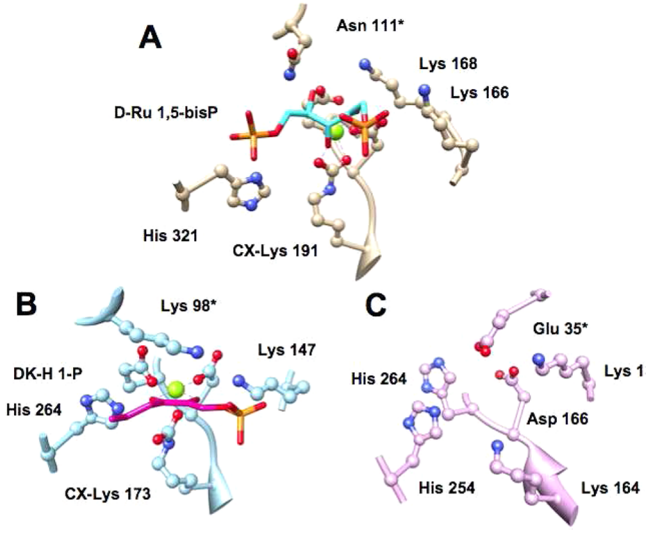

Figure 10. Comparison of the active sites of RuBisCO from *R. rubrum* (A), "enolase" from *G. kaustophilus* (B), and the presumed MTRu 1-P 1,3-isomerase from *Rh. palustris* (C). Details are provided in the text.

RuBisCO, the carboxylated Lys 191 (CX-Lys 191) is the general base for abstraction of the 3-proton as well as a ligand for the essential Mg^{2+} (Lys 166 and 168 are required for enolization; His 321 catalyzes hydration[2]). In "enolase", Lys 98* (from the symmetry-related polypeptide in the obligate dimer) is the general base for abstraction of the 1-proton, the carboxylated Lys 173 (CX-Lys 173) is a ligand for the essential Mg^{2+},[1] and Lys 147 is conserved in all RLPs. The 1,3-isomerase is not activated; i.e., Lys 164 is not carboxylated, nor does the structure contain Mg^{2+}. Asp 166 and His 264 are likely ligands for Mg^{2+}, although no biochemical or structural data are available to support this proposal. Glu 35* (from the symmetry-related polypeptide in the obligate dimer) is positioned to be a general acid–base catalyst as discussed in the next paragraph.

Although no structure–function studies have been performed with the 1,3-isomerase from *Rh. palustris* or *R. rubrum*,[13] a Glu residue (Glu 35 in *Rh. palustris* and Glu 32 in *R. rubrum*) conserved in all members of the 1,3-isomerase family, but not in either RuBisCOs or "enolases", is located on the opposite face of the active site from the carboxylated Lys. The curious, but unexplained, promiscuity of 1,3-isomerase to generate a 3:1 mixture[13] of MTXu 5-P that is the precursor of DXP in the MTA-isoprenoid shunt (Figure 3B) and MTRu 5-P that is a "dead-end" metabolite[11] may reflect the absence of a general acid to protonate carbon-2 of the Mg^{2+}-stabilized enediolate anion in the initial 1,2-proton transfer reaction initiated by the carboxylated Lys.[13] As a result, a mixture of diastereomeric ("racemic") 5-methylthio-3-ulose 1-phosphates is produced by release of the enediolate anion to solvent (only the "productive" diastereomer is shown in Figure 1C). Both diastereomers are then substrates for the second 1,2-proton transfer reaction in which the observed 3:1 mixture of MTXu 5-P and MTRu 5-P products is generated; in this 1,2-proton transfer reaction, the conserved Glu is appropriately positioned to function as the general base that abstracts the 4-proton from both diastereomers of the 3-ulose intermediate and, also, the general acid that protonates carbon-3 of the mixture of enediolate anion intermediates. Thus, even in the absence of experimental studies, we conclude on the basis of stereochemical and structural considerations that the mechanism of the reaction catalyzed by 1,3-isomerase requires general acid–base catalysts on both faces of the active site. Consequently, as for the "enolase" reaction, we do not expect RuBisCO to possess the general acid–base catalysts necessary for the 1,3-isomerase reaction. Therefore, the lack of promiscuity observed both in vitro and in vivo is expected.

Requirements for Discovering Promiscuity for Enzymes in MTA Recycling. Our experiments demonstrate that the presumed nonphysiological functional promiscuity documented for RuBisCOs does not extend to the "enolase" and 1,3-isomerase functions that are catalyzed by the functionally assigned RLPs, at least for RuBisCO from *R. rubrum*. As we have discussed, we are not surprised, given the different stereochemical requirements for proton abstraction in the carboxylation reaction catalyzed by RuBisCO and the proton transfer reactions catalyzed by "enolase" and 1,3-isomerase; i.e., the abstraction of spatially remote protons requires structurally distinct general basic catalysts, not just the carbamate oxygen of the carboxylated Lys residue that is conserved in both RuBisCOs and RLPs.[1]

In the case of the functionally diverse enolase superfamily, promiscuity is observed; however, promiscuity is the result of relaxed substrate specificities that present diverse substrates to a conserved set of active site acid–/base catalysts.[22] Indeed, in vitro experiments have provided support for the hypothesis that natural divergent evolution of function can occur by successive point substitutions that provide enhanced selective advantage by incremental changes in substrate specificity.[23−25] However, for the reactions catalyzed by RuBisCO, DK-MTP "enolase", and MTRu 1-P 1,3-isomerase, the active site acid–base catalysts

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are not conserved, so it is unlikely that any of these enzymes can be promiscuous for the other reactions.

Our recent discovery that MTA recycling can occur by (at least) two different pathways, one involving genuine methionine salvage from MTA (Figure 3A)[10] and the second liberating the 5′-methylthio group of MTA as methanethiol in the MTA-isoprenoid shunt that can be captured by *O*-acetylhomoserine sulfhydrylase to generate methionine (Figure 3B),[11] complicates the interpretations of studies of the ability of RuBisCOs to complement mutants deficient in MTA recycling.[10,21,26] Further difficulties are suggested by the observation that under anaerobic conditions a 1,3-isomerase mutant, but not a RuBisCO mutant, of *R. rubrum* could utilize MTA as the sole sulfur source. As expected, the double mutant could not utilize MTA; however, when the double mutant was transformed with a plasmid encoding the RuBisCO, the MTA utilization phenotype was restored.[21] One explanation is that *R. rubrum* has another pathway for MTA utilization, i.e., neither of those in Figure 3, that recovers at least the methylthio group of MTA for sulfur utilization and that the RuBisCO is involved in this pathway.

CONCLUSIONS

Our experiments do not provide evidence that RuBisCO from *R. rubrum* can catalyze either the DK-MTP 1-P "enolase" or the MTRu 1-P 1,3-isomerase reaction, the two functions now established for RLPs. Perhaps other RuBisCOs are promiscuous for these reactions and/or for reactions catalyzed by RLPs that have not yet been functionally assigned, but until such promiscuity is discovered, few insights into Nature's strategies for divergent evolution of function in the RuBisCO superfamily are available.

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Notes

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ABBREVIATIONS

RuBisCO, D-ribulose 1,5-bisphosphate carboxylase/oxygenase; 3-PGA, 3-phosphoglycerate; RLP, RuBisCO-like protein; MTA, 5′-methylthioadenosine; MTR, 5-methylthio-D-ribose; MTR 1-P, 5-methylthio-D-ribose 1-phosphate; MTRu 1-P, 5-methylthio-D-ribulose 1-phosphate; DK-MTP 1-P, 2,3-diketo-5-methylthiopentane 1-phosphate; HK-MTP 1-P, 2-hydroxy-3-keto-5-methylthiopent-1-ene 1-phosphate; DHK-MTP, 1,2-dihydroxy-3-keto-5-methylthiopent-1-ene; KMTB, 2-keto-4-methylthiobutyrate; MTRu 5-P, 5-methylthio-D-ribulose 5-phosphate; MTXu 1-P, 5-methylthio-D-xylulose 5-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate.

ADDITIONAL NOTE

[a]Although the enzyme activity that accomplishes the keto−enol tautomerization reaction in the methionine salvage pathway has long been termed an "enolase", the usual name for this reaction is "tautomerase". Given our interest in the members of the mechanistically diverse enolase superfamily whose members catalyze reactions that are initiated by abstraction of a proton from a carbon adjacent to a carboxylate group, including the authentic enolase in glycolysis, we would prefer not to use "enolase" to describe the keto−enol tautomerization reaction in the methionine salvage pathway. However, given the history, we compromise by using "enolase" (in quotation marks) to refer to the enzyme in the methionine salvage pathway that catalyzes the keto−enol tautomerization reaction (reproduced from ref 1).

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