Mechanistic Diversity in the RuBisCO Superfamily: A Novel Isomerization Reaction Catalyzed by the RuBisCO-like Protein from *Rhodospirillum rubrum*[†]

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ABSTRACT: Some homologues of D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) do not catalyze carboxylation and are designated RuBisCO-like proteins (RLPs). The RLP from *Rhodospirillum rubrum* (gi: 83593333) catalyzes a novel isomerization reaction (overall 1,3-proton transfer reaction; likely, two 1,2-proton transfer reactions) that converts 5-methylthio-D-ribulose 1-phosphate to a 3:1 mixture of 1-methylthioxylulose 5-phosphate and 1-methylthioribulose 5-phosphate. Disruption of the gene encoding the RLP abolishes the ability of *R. rubrum* to utilize 5′-methylthioadenosine as a sole sulfur source, implicating a new, as-yet-uncharacterized, pathway for sulfur salvage.

D-Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes competing carboxylation and oxygenation reactions, the former yielding two molecules of 3-phosphoglycerate (3-PGA) and the latter one molecule of 3-PGA and one molecule of 2-phosphoglycolate (Figure 1A). Both are initiated by abstraction of the substrate's 3-proton to yield a Mg^{2+}-stabilized enolate that partitions between carboxylation and oxygenation. After enolization, carboxylation and C−C bond cleavage occur via sequential formation of several intermediates (*1*). The mechanistic complexity provides the opportunity for competing side reactions, including epimerization to yield D-xylulose 1,5-bisphosphate, isomerization (1,2-proton transfer) to yield 3-ketoarabinitol 1,5-bisphosphate, and elimination of phosphate to yield 1-deoxy-D-*glycero*-2,3-pentdiulose 5-phosphate (*2, 3*).

Given this promiscuity, it is not surprising that the active site architecture of RuBisCO is used to stabilize enolate intermediates in other reactions. The genomes of eubacteria and archaea encode homologues of RuBisCO that lack residues essential for carboxylation; these are designated "RuBisCO-like proteins" (RLPs) (*4*−*7*). Like RuBisCOs, the structurally characterized RLPs are dimers of bidomain "large" polypeptides; the active sites are located at the interface of the N-terminal α+β domain of one polypeptide and the C-terminal $(\beta/\alpha)_8$-barrel domain of the second polypeptide (*6, 8*−*10*). The active sites of RuBisCOs and many, but not all, RLPs contain a signature KDDE motif at the end of the second β-strand of the barrel domain, where the carbamoyl group of the carboxylated K and the carboxylate groups of the DE sequence are ligands for an essential Mg^{2+}. The C-terminal $(\beta/\alpha)_2$-quarter barrel provides the binding site for the 1-phosphate group of ketose substrates. To date, all RLPs lack one of the Lys residues (homologues of Lys 177 in spinach RuBisCO) required for carboxylation. In many RLPs, homologues of the His and Arg residues that form a binding site for the distal 5-phosphate (His 295 and Arg 327 in spinach RuBisCO) are replaced with hydrophobic residues, suggesting that the substrates are ketose 1-phosphates with distal hydrophobic structures (vide infra).

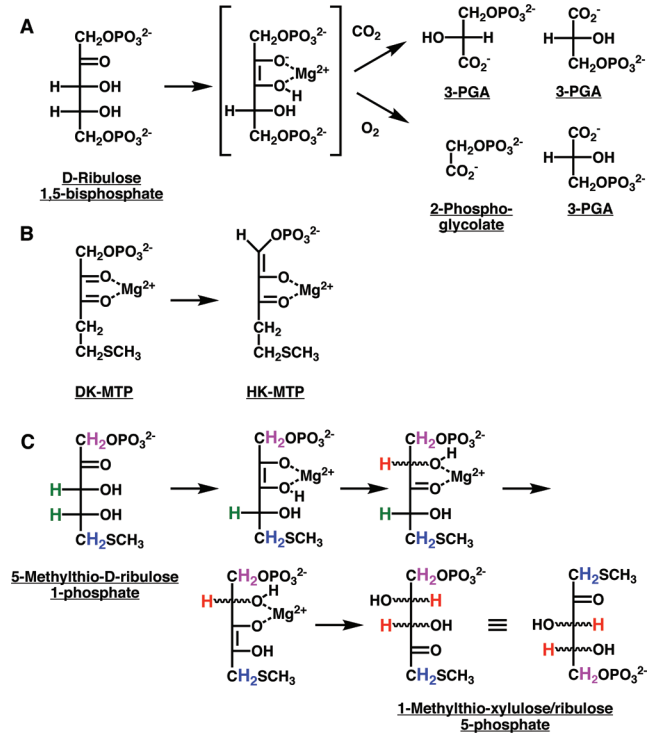

FIGURE 1: Reactions catalyzed by members of the RuBisCO superfamily. (A) RuBisCO carboxylation and oxygenation. (B) DK-MTP tautomerase. (C) RLP from *Rhodospirillum rubrum*.

If the structural strategies for divergent evolution in the RuBisCO superfamily are to be elucidated, the functions of the RLPs must be established. However, functional assignment of unknown proteins discovered in genome projects is

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a challenge (*11, 12*). In the case of the RLPs encoded by species of bacilli, genome context provided sufficient information for deduction of their function (*5, 13*): several of their genes are colocalized in operons with genes encoding enzymes in the methionine salvage pathway [conversion of 5′-methythioadenosine (MTA) to methionine] with the exception of an orthologue of the previously characterized tautomerase that catalyzes the conversion of 2,3-diketo-5-methylthiopentane 1-phosphate (DK-MTP) to 2-hydroxy-3-keto-5-thiomethylpent-1-ene 1-phosphate (HK-MTP) (Figure 1B) (*14*). Subsequent biochemical analyses confirmed that these RLPs are, in fact, analogues of the previously characterized tautomerases. Mechanistic and structural characterization of the tautomerase from *Geobacillus kaustophilus* revealed that (1) Lys 98 in the N-terminal $\alpha+\beta$ domain of the symmetry-related polypeptide is the base that abstracts the 1*S* proton and (2) the hydrophobic 5-methylthio group is located in a hydrophobic pocket (Pro and Leu) that replaces the cationic binding pocket for the 5-phosphate group of D-ribulose 1,5-bisphosphate in RuBisCO (*10*).

R. rubrum encodes an RLP (gi:83593333) that is a member of an uncharacterized family of RLPs that contains a KDDH motif instead of the signature KDDE motif (*6, 7*); like the tautomerases, the sequences contain hydrophobic residues (Pro and Ile/Val) for the formation of a hydrophobic binding pocket for the distal portion of the substrate. *R. rubrum* is able to utilize MTA as a sulfur source, although some of the genes encoding a putative methionine salvage pathway (with a DK-MTP tautomerase) have not yet been identified via homology searches. Disruption of the gene encoding the RLP abolishes the ability to utilize MTA as a sulfur source, establishing the role of this RLP in a sulfur salvage pathway (J. Singh and F. R. Tabita, unpublished results). Perhaps the unidentified enzymes in the methionine salvage pathway are replaced by analogues, or an as-yet-uncharacterized sulfur salvage pathway is present in this organism.

The reaction catalyzed by DK-MTP tautomerase can be asasyed by either (1) an increase in absorbance at 278 nm (formation of the conjugated enol) using a stable analogue of DK-MTP as a substrate or (2) formation of a downfield doublet for the enol (carbon-1) proton in the ^{1}H NMR spectrum when DK-MTP is used as the substrate. When the RLP from *R. rubrum* was used in these assays, tautomerization could not be detected; we previously demonstrated that the RLPs (tautomerases) from *Bacillus subtilis* and *G. kaustophilus* are active in these assays (*10*). Therefore, the RLP from *R. rubrum* does not participate in the usual methionine salvage pathway.

However, as assessed by ^{1}H NMR in D_2O, the RLP catalyzes the rapid conversion of 5-methylthio-D-ribulose 1-phosphate (0.5 s^{-1} using 10 mM substrate) to a 3:1 mixture of products. The only product resonances (Figure 2B) are those of (1) two methylthio groups (1.9 ppm, 3:1 ratio, a total of three protons) and (2) two methylene groups coupled to ^{31}P (3.6−3.8 ppm, two protons, magenta line) and shifted upfield from those of the ^{31}P-coupled methylene protons of carbon-1 of the substrate (4.5 ppm, Figure 2A). With respect to Figure 1C, these resonances account for five of the nine carbon-bound protons associated with the substrate, with four (green and blue, vide infra) exchanging with solvent during the incubation.

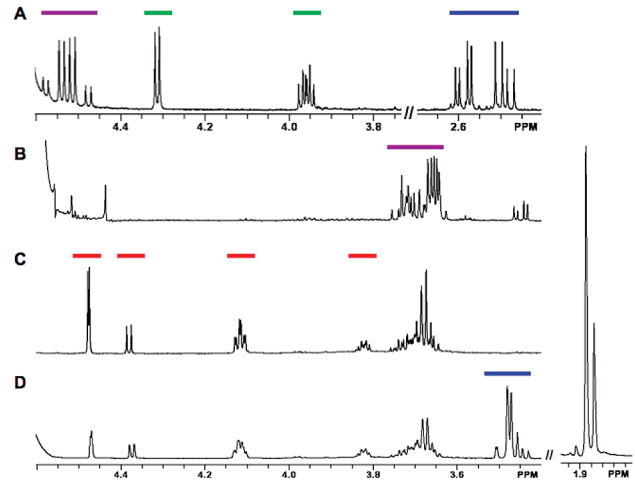

FIGURE 2: ^{1}H NMR spectra of the substrate and product. (A) 5-Methylthio-D-ribulose 1-phosphate in D_2O. (B) Reaction in D_2O and NMR in D_2O. (C) Reaction in H_2O and NMR in D_2O. (D) Reaction in H_2O and NMR in H_2O.

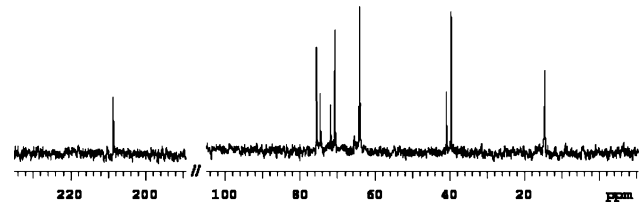

FIGURE 3: ^{13}C NMR spectrum of the product from reaction in H_2O and NMR in H_2O (^{1}H NMR spectrum in Figure 2D).

A reaction was conducted in H_2O, and the enzyme and solvent were removed. A negative ion mass spectrum was obtained; the mass, 259 amu, is identical to that for the substrate, requiring that the product be obtained by an isomerization reaction.

A ^{1}H NMR spectrum of this protiated product dissolved in D_2O revealed the two additional pairs of coupled resonances that integrated to a total of two protons (red in Figures 1C and 2C), each pair a doublet and a doublet of triplets in the expected 3:1 ratio; in addition, the methylene protons coupled to ^{31}P showed evidence for additional ^{1}H−^{1}H coupling. A ^{13}C spectrum of the same sample revealed five pairs of resonances, each present in a 3:1 ratio (Figure 5 of the Supporting Information). Curiously, on the basis of the mass of the product, the resonances associated with two protons and one carbon are “missing” from these NMR spectra. The missing resonances can be explained by hydrogens that when exchanged with deuterium weaken the signal of the directly bonded ^{13}C by loss of NOE, ^{2}H−^{13}C coupling, and other factors.

^{1}H and ^{13}C NMR spectra were obtained following lyophilization of this sample to remove the D_2O and dissolution in H_2O. The ^{1}H NMR spectrum revealed the resonances of additional methylene groups (3.4−3.5 ppm; 3:1 ratio; two protons, blue in Figures 1C and 2D), so in this spectrum, the resonances associated with the nine expected protons in each of the two isomerized products (3:1) are present. The ^{13}C NMR spectrum revealed pairs of resonances (in a 3:1 ratio) at appropriate chemical shifts for the six expected carbons (Figure 3). The appearance of the additional resonances observed in H_2O but not D_2O is not associated with the RLP: the enzyme was removed immediately after

the enzyme-catalyzed reaction in H_2O. Therefore, the RLP-catalyzed reaction involves exchange of two substrate-derived protons with solvent (red in Figure 1C); the product undergoes facile uncatalyzed exchange of the two additional protons with solvent (blue in Figure 1C).

Analyses of the chemical shifts and coupling networks in the ^{1}H NMR spectrum of the fully protiated mixture of products allow the conclusion that the RLP catalyzes the conversion of 5-methylthio-D-ribulose 1-phosphate to a 3:1 mixture of 1-methylthioxylulose 5-phosphate and 1-methylthioribulose 5-phosphate (Figure 1C; the numbering is "inverted" in the products to simplify the nomenclature and, also, relate the structures to likely, but currently unidentified, downstream metabolic transformations; the absolute configurations of the products have not yet been established). Inspection of the structures of the substrate and products suggests two successive 1,2-proton transfer reactions, thereby explaining the absence of resonances for the 3- and 4-protons when the RLP-catalyzed reaction is conducted in D_2O. The 1-methylene groups of the products are susceptible to facile nonenzymatic exchange with solvent, perhaps as the result of intramolecular catalysis by the phosphate group.

That the RLP can catalyze an overall 1,3-proton transfer reaction via two successive 1,2-proton transfer reactions initiated by abstraction of the 3-proton of the substrate is not surprising; RuBisCO analogously initiates its carboxylation reaction. Protonation of the resulting 2,3-enediolate anion on carbon-2 would produce a 3-keto intermediate; interestingly, RuBisCO produces the analogous 3-ketoarabinitol 1,5-bisphosphate as a promiscuous product (*3*). However, that the RLP catalyzes a second 1,2-proton transfer reaction initiated by abstraction of the 4-proton from the 3-keto intermediate to generate a second 3,4-enediolate anion is surprising: bidentate coordination with Mg^{2+} to stabilize both the 2,3- and 3,4-enediolate intermediates is expected to require movement of the Mg^{2+} and/or the substrate on the reaction coordinate, although the same base could catalyze abstraction of a proton from carbon-3 of the substrate and carbon-4 of the presumed 3-keto intermediate. Continuous monitoring of the reaction by ^{1}H NMR does not reveal transient accumulation of the 3-keto intermediate, suggesting that it remains enzyme-bound during the course of the reaction. A structure of the RLP from *R. rubrum* will be illuminating with respect to understanding the structural basis for generating and stabilizing two different enediolate intermediates.

The rapid rate of this isomerization reaction (0.5 s^{-1} using 10 mM substrate) suggests that it is not an adventitious reaction and that 5-methylthio-D-ribulose 1-phosphate is the correct substrate. Some members of this family of RLPs are encoded by operons that also encode orthologues of 5-methylthio-D-ribose 1-kinase and 5-methylthio-D-ribose 1-phosphate isomerase (enzymes that produce 5-methylthio-D-ribulose 1-phosphate in the methionine salvage pathway), supporting this substrate specificity. The structure of the product of the "double isomerase" would appear to require that MTA salvage in *R. rubrum* does not regenerate methionine from MTA but, perhaps, produces cysteine or a novel metabolite in an as-yet-undiscovered pathway for sulfur salvage.

SUPPORTING INFORMATION AVAILABLE

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

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