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Stereochemical Course of the Reaction Catalyzed by RimO, a Radical SAM Methylthiotransferase

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Supporting Information

ABSTRACT: RimO is a member of the growing radical S-adenosylmethionine (SAM) superfamily of enzymes, which use a reduced [4Fe−4S] cluster to effect reductive cleavage of the 5′ C−S bond of SAM to form a 5′-deoxyadenosyl 5′-radical (5′-dA•) intermediate. RimO uses this potent oxidant to catalyze the attachment of a methylthio group (−SCH_3) to C3 of aspartate 89 of protein S12, one of 21 proteins that compose the 30S subunit of the bacterial ribosome. However, the exact mechanism by which this transformation takes place has remained elusive. Herein, we describe the stereochemical course of the RimO reaction. Using peptide mimics of the S12 protein bearing deuterium at the 3 *pro-R* or 3 *pro-S* positions of the target aspartyl residue, we show that RimO from *Bacteroides thetaiotaomicron* (*Bt*) catalyzes abstraction of the *pro-S* hydrogen atom, as evidenced by the transfer of deuterium into 5′-deoxyadenosine (5′-dAH). The observed kinetic isotope effect on H atom versus D atom abstraction is ∼1.9, suggesting that this step is at least partially rate determining. We also demonstrate that *Bt* RimO can utilize the flavodoxin/flavodoxin oxidoreductase/NADPH reducing system from *Escherichia coli* as a source of requisite electrons. Use of this *in vivo* reducing system decreases, but does not eliminate, formation of 5′-dAH in excess of methylthiolated product.

RimO (ribosomal modification O) catalyzes the post-translational modification of aspartate 89 (D89) of protein S12 to give 3-methylthioaspartate (3-MS-D89).[1] Protein S12 is a component of the 30S subunit of the bacterial ribosome, and the loop on which D89 resides projects into the acceptor site where aminoacyl tRNAs bind.[2] The modification itself is not essential, but *Escherichia coli* (*Ec*) that are capable of catalyzing this methylthiolation reaction have a slight growth advantage over those that are not. This growth advantage is believed to be related to enhanced translational fidelity.[1] Two recent X-ray structures of the *Thermotoga maritima* (2.3−2.5 Å) and *Ec* (2.4 Å) ribosome showed electron density for 3-MS-D89, and in the *Ec* structure the absolute stereochemistry at C3 was observed to be *R*.[3,4] The methylthio group points toward the 6-oxo group of N^7-methylguanosine 527, a modified nucleobase in 16S rRNA; however, the purpose of the interaction between the modified protein residue and the nucleobase is unknown.

As a member of the radical SAM (RS) superfamily of enzymes, RimO uses a $[4Fe{-}4S]^{+}$ cluster to promote the reductive cleavage of the 5′ C−S bond of SAM to form a 5′-deoxyadenosyl 5′-radical (5′-dA•).[5−7] This potent oxidant has been suggested to abstract a hydrogen atom (H•) from C3 of D89 to activate it for methylthiolation, although this particular step of the reaction has never been demonstrated.[1,6,7] In addition to the [4Fe−4S] cluster that participates in the reductive cleavage of SAM (RS cluster), RimO harbors an additional [4Fe−4S] cluster (auxiliary cluster) in its N-terminal region. The auxiliary cluster was previously believed to be sacrificed during the first phase of the reaction to provide the inserted sulfur atom[6] in a mechanism analogous to those proposed for the RS sulfurtransferases, lipoyl synthase (LipA),[8−12] and biotin synthase (BioB).[13−17] In the second phase of RimO catalysis, the inserted sulfur atom was proposed to undergo methylation by a canonical SAM-dependent S_N2 mechanism.[6] Recent studies suggest, however, that RimO and the related enzyme, MiaB, catalyze the initial synthesis of a methylthio group that is most likely attached externally to the auxiliary Fe/S cluster and that the entire methylthio group is subsequently transferred intact to C3 of the aspartyl residue via radical chemistry.[18,19] These observations indicate that RimO and MiaB are members of an emerging subclass of RS enzymes that, within a single active site, activate SAM both for methyltransfer and for generation of a 5′-dA•.[20,21]

Although RimOs both from *Escherichia coli* (*Ec*)[6] and from *Thermotoga maritima* (*Tm*)[7] have been characterized, *Tm* RimO is better behaved and exhibits significantly greater turnover. However, the need to use the artificial reductant, sodium dithionite, in activity determinations of *Tm* RimO induces production of SAH and 5′-dAH that was believed to be uncoupled from product formation. In this study, we show that RimO from the gut bacterium, *Bacteroides thetaiotaomicron*, can utilize the *Ec* flavodoxin/flavodoxin oxidoreductase/NADPH (Fld/FldR/NADPH) reducing system as a source of electrons for catalysis. We also determine the stereochemistry of H•

Scheme 1. Reaction Catalyzed by RimO, with the Absolute Configuration of the Carbon Atom To Which the Methylthio Group Is Appended Indicated in Green as *3R*

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abstraction from D89 of the S12 protein through the use of chemoenzymatically synthesized deuterated isotopomers at C3 of an aspartate residue incorporated into a 13-amino acid peptide mimic (13-mer) of the S12 protein. We observe transfer of deuterium into 5′-deoxyadenosine only from the 13-mer containing [(2*S*,3*S*)-2,3-$^2H_2$]-aspartate and not [(3*R*)-3-$^2H_1$]-aspartate, indicating that H$^\bullet$ abstraction is indeed stereoselective, and that insertion of the $-SCH_3$ group occurs with inversion of configuration at C3. The apparent kinetic isotope effect associated with H versus D atom abstraction by the 5′-dA$^\bullet$ is ∼1.9, indicating that H atom abstraction is at least partially rate-limiting.

In previous studies of *Ec* and *Tm* RimOs, dithionite was used as the source of requisite electrons for catalysis. *Tm* RimO catalyzed methylthiolation in the presence of dithionite, but not in the presence of the *Ec* Fld/FldR/NADPH reducing system, while *Ec* RimO did not exhibit appreciable activity with either reductant. The use of the chemical reductant in the *Tm* RimO reaction, however, was believed to induce abortive cleavage of SAM, as evidenced by production of 5′-dAH and SAH that were in excess over the methylthiolated product.[7,18,19] The *Tm* genome does not appear to encode flavodoxins but does encode five ferredoxins, one or more of which might function to deliver electrons to RS enzymes. However, because of our interest in RS enzymes from gut microbiota and the desire to work with enzymes that function optimally closer to ambient temperature, we chose to study RimO from the major human gut bacterium, *Bacteroides thetaiotaomicron* (*Bt*), which does contain an annotated flavodoxin gene. *Bt* RimO is 35% identical to *Ec* RimO and 39% identical to *Tm* RimO, while *Bt* flavodoxin is 37% identical to *Ec* flavodoxin. The gene encoding *Bt* RimO was coexpressed with plasmid pDB1282, and its protein product was isolated as previously described for *Tm* and *Ec* RimOs.[6,19]

When *Bt* RimO was incubated under turnover conditions in the presence of the Fld/FldR/NADPH reducing system and a 13-aa peptide corresponding to residues 83−95 of the *Bt* S12 protein, formation of SAH (m/z = 385.0), 5′-dAH (m/z = 252.1), and methylthiolated peptide, **3-MS-1** (m/z = 760.1), was observed by LC/MS (Figure 1). Interestingly, however, the final concentrations of SAH and 5′-dAH were 1.1- to 2.5-fold higher than that of the methylthiolated product, which is similar to that observed when assays were conducted using dithionite as the requisite source of electrons. To determine initial rates of formation for SAH, 5′-dAH, and **3-MS-1**, the amplitudes of each of the corresponding curves were multiplied by the first-order rate constants obtained from fits of the data to a single-exponential equation, resulting in rates of 19.7 ± 0.68 μM min^{-1} (SAH) 6.03 ± 0.01 μM min^{-1} (5′-dAH), and 3.95 ± 0.01 μM min^{-1} (3-MS-1). These results suggest that methylation of RimO by SAM takes place relatively rapidly as compared to formation of 5′-dAH and product and that the *Ec* Fld/FldR/NADPH reducing system is indeed capable of delivering electrons to Bt RimO for catalysis. Interestingly, while formation of 5′-dAH, even in the presence of the *in vivo* reducing system, is in excess of methylthiolated product, reactions conducted in ∼90% D_2O result in no 5′-dAD above natural abundance. This observation suggests that any 5′-dA$^\bullet$ that does not lead to the correct product abstracts an enzyme- or substrate-derived H atom that is not from a solvent exchangeable site (SI Figure 7).

To determine the stereoselectivity of H atom abstraction, deuterium-containing isotopomers (*pro-R* or *pro-S*) at C3 of aspartate were incorporated into peptide substrates, which were subsequently used in the RimO reaction. The synthesis of the 3-

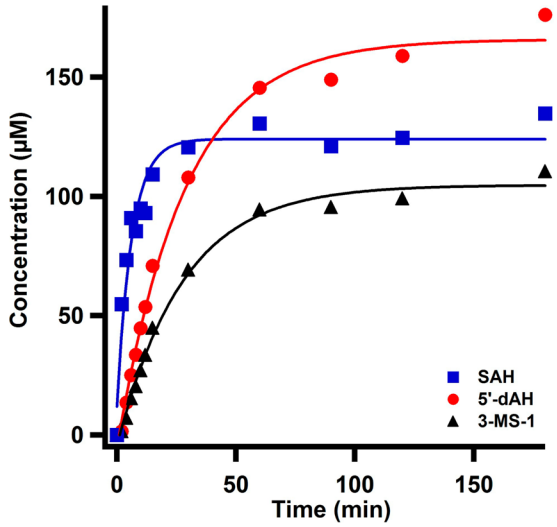

Figure 1. *Bt* RimO-catalyzed time-dependent formation of SAH, 5′-dAH, and methylthiolated product (**3-MS-1**) with the *Ec* Fld/FldR/NADPH reducing system. The reaction was conducted as described in the Supporting Information and contained 100 μM *Bt* RimO, 3 mM SAM, 3 mM peptide (**1**), 200 μM Fld, 50 μM FldR, 3 mM NADPH, and 50 mM Na-HEPES pH 7.5.

pro-R and 3-*pro*-S deuterated substrates followed the strategies described by Young et al.[22] and Richards et al. (SI Scheme 1),[23] which exploit the ability of aspartate ammonia-lyase (AAL) to catalyze the stereoselective incorporation of deuterium from D_2O into the C3 *pro-R* position of aspartate when incubated with fumarate and excess ammonium chloride to afford (2*S*,3*R*)-3-[$^2H_1$] aspartate. Similarly, AAL catalyzes the stereoselective incorporation of a proton in the C3 *pro-R* position of aspartate and, when incubated with [2,3-$^2H_2$]-fumarate and ammonium chloride in H_2O, affords (2*S*,3*S*)-[2,3-$^2H_2$] aspartate.[23] We confirmed the selective deuterium incorporation in both labeled aspartates by ^{1}H NMR (SI Figures 2 and 3) after converting their side chain carboxylic acids to *tert*-butyl esters and protecting their amine groups with Fmoc. Displayed in panels A, B, and C of Figure 2 are the expanded regions (2.4 to 4.7 ppm) of the ^{1}H NMR spectra of the unlabeled, *pro-R* labeled, and *pro-S* labeled protected aspartates, respectively, where the C2 and C3 proton

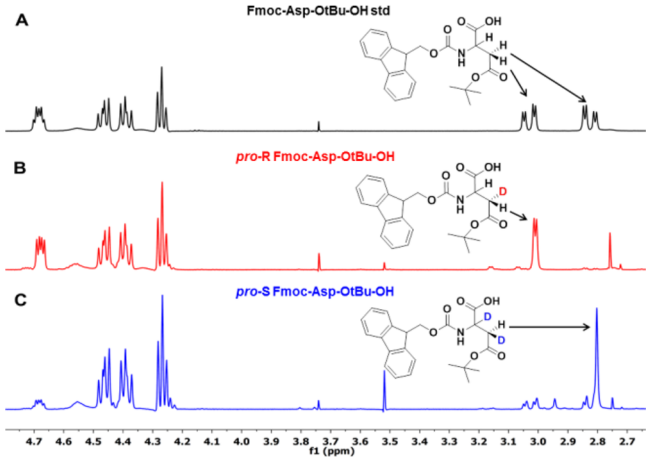

Figure 2. ^{1}H NMR spectra of unlabeled (A), *pro-R* labeled (B), and *pro-S* labeled (C) aspartate with its amino and β-carboxylic acid moieties protected with Fmoc and *tert*-butyl ester groups, respectively.

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signals are observed. The C3 hydrogens of unlabeled Fmoc-Asp-β-O*t*Bu in panel A exhibit both geminal coupling and vicinal coupling to the hydrogen on C2, resulting in two sets of doublets of doublets. The doublet of doublets corresponding to the *pro-R* hydrogen is centered at 2.77 ppm, while that of the *pro-S* hydrogen is observed at 2.98 ppm. In panel B, the replacement of the *pro-R* hydrogen with deuterium results in the disappearance of the doublet of doublets at 2.77 ppm. Additionally, one of the doublets at 2.98 ppm that was present due to geminal coupling is now absent, confirming that the *pro-R* deuterium was indeed retained. Similarly, in panel C, the replacement of the *pro-S* hydrogen at C3 and the hydrogen at C2 with deuterium eliminated geminal and vicinal proton coupling and resulted in the collapse of the doublet to a single peak at 2.77 ppm, corresponding to the C3 *pro-R* hydrogen. The absence both of the proton signal at 2.98 ppm and of proton coupling confirmed the presence of deuterium at C2 and in the *pro-S* position at C3.

Catalysis by RimO is believed to involve H atom abstraction from C3 of aspartate by a 5′-dA$^{\bullet}$ generated from the reductive cleavage of SAM. To determine the overall stereochemical course of the RimO reaction, the stereoselectively labeled aspartates were appropriately protected for solid phase peptide synthesis and incorporated at the target position of the 13-mer peptide derived from the *Bt* S12 protein. The peptide containing the C3 *pro-R* aspartate (**2**) and the peptide containing the C3 *pro-S* aspartate (**3**) were used as substrates in reactions with *Bt* RimO to determine which of the two peptides supports the formation of 5′-deoxyadenosine enriched with deuterium (5′-dAD), thereby indicating which of the H atoms attached to C3 is abstracted. Panels A, B, and C of Figure 3 show the time-dependent formation of 5′-dAH, 5′-dAD, and methylthiol-containing product (**3-MS**) obtained using peptides **1**, **2**, or **3**, respectively. Formation of 5′-dAD is only observed with peptide **3** containing the C3 *pro-S*-labeled aspartate, indicating that the 5′-dA$^{\bullet}$ abstracts the *pro-S* H atom from the substrate and that the overall RimO reaction proceeds with inversion of configuration. A primary kinetic isotope effect of ∼1.9 was calculated from the ratios of the rates of 5′-dAH formation with peptide **1** (5.41 ± 0.01 μM min^{-1}) and 5′-dAD + 5′-dAH formation with peptide **3** (2.88 ± 0.01 μM min^{-1}), indicating that H atom abstraction is at least partially rate-limiting. Furthermore, there appears to be a substantial secondary isotope effect (∼1.4) in the reaction using the 3-*pro-R*-labeled substrate (**2**), given the rates of 5′-dAH formation with peptide **1** (5.41 ± 0.01 μM min^{-1}) versus peptide **2** (3.7 ± 0.01 μM min^{-1}).

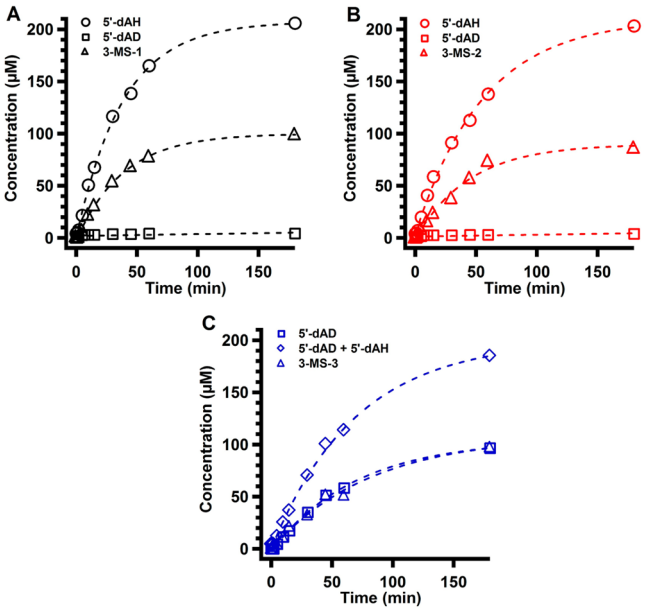

Figure 3. *Bt* RimO catalyzed reactions at 37 °C in the presence of *Ec* Fld/FldR/NADPH, SAM, and peptides **1** (A), **2** (B), or **3** (C). The reactions were conducted as described in the Supporting Information and contained 100 μM *Bt* RimO, 3 mM SAM, 3 mM peptide (**1**, **2**, or **3** as indicated), 200 μM Fld, 50 μM FldR, 3 mM NADPH, and 50 mM Na-HEPES pH 7.5.

The source of sulfur that is inserted in the RimO methylthiolation reaction has yet to be definitively determined; however, it has been proposed that the auxiliary N-terminal [4Fe−4S] cluster serves this role[6] or serves as a binding site for a sulfide species that is methylated and subsequently inserted into the substrate in a radical-dependent process.[18,19] To determine whether methionine, a byproduct formed during the reductive cleavage of SAM, is used by RimO as a source of sulfur in the methylthio group, the concentrations of methionine and 5′-dAH formed in a reaction of *Bt* RimO incubated under turnover conditions with **1** and the *Ec* Fld/FldR/NADPH reducing system were quantified and compared (SI Figure 6). Methionine and 5′-dAH were formed in a 1:1 ratio, thereby ruling out methionine as a source of sulfide/methylthio group for the RimO reaction.

Previous studies of *Tm* RimO conducted by two different laboratories shed light on the mechanism by which this enzyme catalyzes methylthiolation of D89.[6,7,18,19] Contrary to the initial proposed mechanism, based on the mechanisms of the RS sulfurtransferases BioB[15,24−29] and LipA,[11,30] in which the auxiliary clusters of these enzymes were shown to be the sacrificial source of sulfide, RimO has been shown to synthesize an $-SCH_3$ group that is presumably bound to the unique Fe ion of its auxiliary cluster.[18,19] Subsequent generation of the 5′-dA$^{\bullet}$ for H atom abstraction, now known from this study to be the *pro-S* H atom, generates a substrate-based radical with which the synthesized $-SCH_3$ group presumably combines to form the methylthiolated product. Although the details of the attachment of the methylthio group onto the substrate remain elusive, the determination of both the stereospecificity of H atom abstraction and the absolute configuration at C3 of 3-MS-D89 allows us to conclude that $-SCH_3$ insertion occurs with inversion of configuration. Together, these results make RimO the third RS enzyme for which the stereochemical outcomes of H atom abstraction and sulfur/methylthio group insertion is known.[12,17]

The finding that the *Ec* Fld/FldR/NADPH reducing system acts as a competent source of reducing equivalents for the *Bt* RimO reaction led us to believe that it would allow demonstration of the expected product ratios of 1:1:1 (methylthiolated product/SAH/5′-dAH). Surprisingly, 5′-dAH and SAH formation in excess of product was still observed, mirroring the results that we, and others, have reported for *Tm* RimO.[7,18,19] In previous studies of the *Tm* RimO reaction in which dithionite was used, formation of both SAH and 5′-dAH in 2-fold or greater excess of product was observed, which was attributed to abortive cleavage of SAM, a known side reaction of RS enzymes.[7,18,19] The use of the Ec Fld/FldR/NADPH reducing system did decrease the amount of SAH and 5′-dAH formed per methylthiolated product; however, abortive cleavage still resulted. Reactions conducted in D_2O demonstrated that solvent and solvent exchangeable H atoms do not quench any 5′-dA$^{\bullet}$ formed productively or abortively, suggesting that

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abstraction of an H atom derived from the RimO polypeptide or the peptide substrate occurred. Future studies will address the questions concerning the overall stoichiometry of reactants and products and the source of sulfide in the methylthiolation reaction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b11035.

Experimental methods, synthetic details, and additional spectra and figures (PDF)

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Notes

The authors declare no competing financial interest.

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