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Substrate-Dependent Cleavage Site Selection by Unconventional Radical *S*-Adenosylmethionine Enzymes in Diphthamide Biosynthesis

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Supporting Information

ABSTRACT: *S*-Adenosylmethionine (SAM) has a sulfonium ion with three distinct C-S bonds. Conventional radical SAM enzymes use a [4Fe-4S] cluster to cleave homolytically the $C_{5',adenosine}$-S bond of SAM to generate a 5′-deoxyadenosyl radical, which catalyzes various downstream chemical reactions. Radical SAM enzymes involved in diphthamide biosynthesis, such as *Pyrococcus horikoshii* Dph2 (*Ph*Dph2) and yeast Dph1-Dph2 instead cleave the $C_{\gamma,Met}$-S bond of methionine to generate a 3-amino-3-carboxylpropyl radical. We here show radical SAM enzymes can be tuned to cleave the third C-S bond to the sulfonium sulfur by changing the structure of SAM. With a decarboxyl SAM analogue (dc-SAM), *Ph*Dph2 cleaves the C_{methyl}-S bond, forming 5′-deoxy-5′-(3-aminopropylthio) adenosine (dAPTA, **1**). The methyl cleavage activity, like the cleavage of the other two C-S bonds, is dependent on the presence of a $[4Fe\text{-}4S]^{+}$ cluster. Electron-nuclear double resonance and mass spectroscopy data suggests that mechanistically one of the S atoms in the [4Fe-4S] cluster captures the methyl group from dc-SAM, forming a distinct EPR-active intermediate, which can transfer the methyl group to nucleophiles such as dithiothreitol. This reveals the [4Fe-4S] cluster in a radical SAM enzyme can be tuned to cleave any one of the three bonds to the sulfonium sulfur of SAM or analogues, and is the first demonstration a radical SAM enzyme could switch from an Fe-based one electron transfer reaction to a S-based two electron transfer reaction in a substrate-dependent manner. This study provides an illustration of the versatile reactivity of Fe-S clusters.

S-Adenosylmethionine (SAM) is a versatile molecule (Figure 1).[1,2] In addition to being a well-known methyl donor in many biological reactions, SAM is cleaved homolytically to generate methionine and a 5′-deoxyadenosyl (5′-dA) radical (Figure 1), which can initiate numerous reactions. Proteins that catalyze these reactions are known as radical SAM enzymes[3,4] and comprise a superfamily of ∼113 000 members[5] that share a conserved three cysteine motif (mostly CxxxCxxC) that binds a [4Fe-4S] cluster. SAM can also be homolytically cleaved by unconventional radical SAM enzymes to generate a 3-amino-3-carboxylpropyl (ACP) radical, used in diphthamide biosynthesis in archaeal and eukaryotic species.[6] For example, the thermophilic archaeon *Pyrococcus horikoshii* diphthamide biosynthesis protein, *Ph*Dph2, transfers the ACP group from SAM to the substrate protein, *P. horikoshii* translation elongation factor 2 (*Ph*EF2).[7,8] *Ph*Dph2 forms a homodimer and each monomer binds a [4Fe-4S] cluster. The yeast Dph1-Dph2 heterodimer complex, an ortholog of the *Ph*Dph2 homodimer, can also cleave SAM and transfer the ACP group to yeast EF2 via the same radical mechanism.[9]

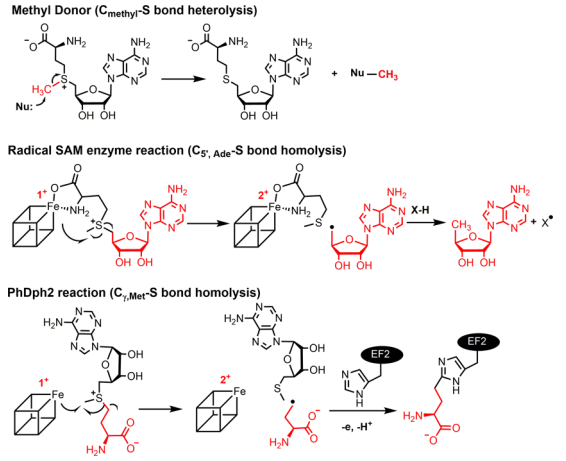

Figure 1. Three categories of C-S bond cleavage reactions of SAM.

When SAM is used as a methyl donor, the C-S bond between the methyl group and sulfonium (C_{methyl}-S bond) is cleaved heterolytically, through nucleophilic attack by the acceptor atom, without a [4Fe-4S] cluster (Figure 1). Although there are several methylases that transfer methyl group from SAM to inert carbon atoms through generation of a 5′-dA radical to initiate the reaction,[10] no radical SAM enzyme has been found to cleave homolytically the C_{methyl}-S bond.

A question in the radical SAM enzyme community is how the radical SAM enzymes control which C-S bond in SAM is cleaved. Here we find *Ph*Dph2 cleaves the C_{Methyl}-S bond of a SAM

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analogue, decarboxyl SAM (dc-SAM), forming 5′-deoxy-5′-(3-aminopropylthio) adenosine (dAPTA, **1**), demonstrating for the first time radical SAM enzymes can cleave all three sulfonium C-S bonds, providing important insight into how different enzymes control the cleavage of SAM.

In conventional radical SAM enzymes, the amino and carboxyl groups of SAM coordinate the unique iron in the cluster.[11] The interaction of SAM with the iron sulfur cluster is critical for activity. We began by synthesizing two SAM analogues to test whether the amino and carboxyl groups of SAM are also important in *Ph*Dph2-catalyzed reaction: deamino SAM (da-SAM) and decarboxyl-SAM (dc-SAM) (Figure 2). Although SAM analogues

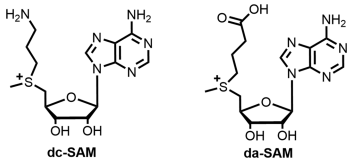

Figure 2. Structure of dc-SAM and da-SAM.

with the adenine changed to other bases have been used to study a radical SAM enzyme NosL,[12] da-SAM and dc-SAM had not. The da-SAM was not a substrate of *Ph*Dph2; we could only detect nonenzymatic decomposition of da-SAM but not *Ph*Dph2-catalyzed cleavage (Figure S1). In contrast, based on HPLC analysis, incubation of dc-SAM with *Ph*Dph2 produced a new compound different from 5′-deoxy-5′-methylthioadenosine (MTA), the product generated in the reaction with natural substrate SAM (Figure 3B). This compound was only produced

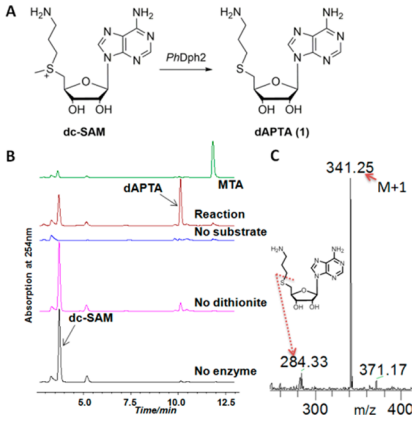

Figure 3. Reaction of dc-SAM with *Ph*Dph2. (A) *Ph*Dph2 converts dc-SAM to dAPTA, **1**. (B) High-performance liquid chromatography showing a new product, different from standard MTA, was generated. (C) ESI-MS of the isolated new peak in panel B. The $[M+1]^+$ of the demethyl product **1** and the fragment peak can be detected.

when *Ph*Dph2 and dithionite were present, suggesting it was an enzymatic reaction product and required the reduced [4Fe-4S] cluster. The rate of this reaction was comparable to that of the natural substrate SAM (Figure S2). The new compound was isolated and characterized by electrospray ionization mass spectrometry (ESI-MS) to be dAPTA, **1**, the demethyl product of dc-SAM (Figure 3C).

The fact that without the carboxyl group of SAM, *Ph*Dph2 could cleave a different C-S bond was very intriguing and surprising. Mechanistically, this showed the amino and carboxyl groups in SAM are important for positioning SAM in the active site of *Ph*Dph2 for the natural enzymatic reaction.

It is known the amino and carboxyl groups of SAM are crucial in conventional radical SAM enzymes.[13] The bidentate interaction of the amino and carboxyl groups of SAM with the unique iron in the [4Fe-4S] cluster facilitates electron transfer from the unique iron to the sulfonium of SAM, resulting in reductive cleavage of SAM and generation of the 5′-dA radical. We thus tested whether conventional radical SAM enzymes would recognize dc-SAM or da-SAM. We incubated dc-SAM or da-SAM with BtrN,[14,15] a radical SAM dehydrogenase involved in the biosynthesis of antibiotic butirosin B. No dc-SAM or da-SAM cleavage product was detected (Figure S3). The result not only highlighted the different reactivities of *Ph*Dph2 and conventional radical SAM enzymes but also showed the amino and carboxyl groups of SAM are important for the reactivities of both types of enzymes.

We further investigated whether the amino group in dc-SAM was important for methyl cleavage by *Ph*DPh2. We synthesized two other SAM analogues (**2** and **3**, Figure 4), with an *m*-

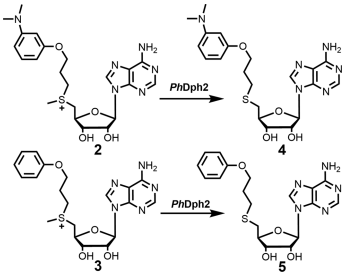

Figure 4. *Ph*Dph2 catalyzes demethylation on SAM analogues **2** and **3**.

dimethylamino-phenoxyl and phenoxyl groups substituting the amino group of dc-SAM. When analogue **2** was incubated with *Ph*Dph2, we detected formation of the demethyl product compound **4**. Similarly, when analogue **3** was incubated with *Ph*Dph2, the demethyl product **5** was also produced (Figure S4). These results suggested the amino group in dc-SAM is not important for this cleavage activity.

To determine the fate of the cleaved methyl group from dc-SAM, we used $^{13}C_{methyl}$-dc-SAM and ^{13}C NMR to monitor the reaction. A new peak around 15 ppm was detected in the reaction, which was absent in the control reaction without dithionite (Figure S5A). The chemical shift indicated a methylated thiol group, likely resulting from the reaction with the DTT molecules in the buffer. This was further confirmed with the detection of Badan (6-bromoacetyl-2-dimethylaminonaphthalene)-derivatized methylated DTT by LC-MS (Figure S5B). When we carried out the same reaction with *Ph*Dph2 and dc-SAM in the absence of DTT, demethylation still occurred, but was much slower. We hypothesized proteins in the reaction could be the methyl acceptor in the absence of DTT. To test this, we tried the reaction of *Ph*Dph2 and $^{14}C_{methyl}$-dc-SAM without DTT in the buffer. $^{14}C_{methyl}$-dc-SAM was prepared in situ by using SAM decarboxylase (SAMDC) and $^{14}C_{methyl}$-SAM before adding *Ph*Dph2. Interestingly, the SAM decarbxylase in the reaction was strongly labeled (Figure 5). The labeling of SAM decarboxylase was dependent on dithionite, suggesting the methylation event was dependent on an active *Ph*Dph2 with the reduced $[4Fe\text{-}4S]^+$ cluster. These results showed both small molecules and proteins could serve as methyl acceptor in the reaction with *Ph*Dph2 and dc-SAM.

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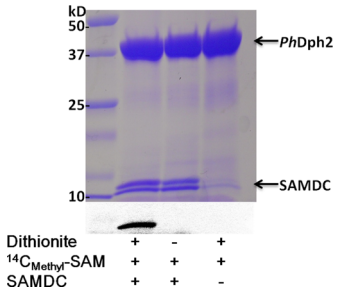

Figure 5. *Ph*Dph2 transfers ^{14}C-methyl group to SAM decarboxylase (SAMDC) from $^{14}C_{methyl}$-dc-SAM. Top shows Coomassie blue-stained gel; bottom shows autoradiography (the full autoradiograph image is shown in Figure S6). Reactions in lanes 1−3 contained *Ph*Dph2. Presence of other reagents indicated below each lane.

To explore the mechanism and search for intermediates in the methyltransfer reaction, we switched to the yeast ortholog Dph1-Dph2 system because the enzyme was active at room temperature, which facilitated experiments designed to capture potential reaction intermediate. Similar to *Ph*Dph2, Dph1-Dph2 cleaved dc-SAM to generate dAPTA and methylated SAMDC (Figure S6). We set up Dph1-Dph2 reaction with dc-SAM without DTT, collected samples at different time points by manually freezing the samples in liquid nitrogen, and examined them by electron paramagnetic resonance (EPR) spectroscopy. A new species with $g = [2.03, 1.98, 1.95]$ accumulated, whereas the original $[4Fe\text{-}4S]^+$ signal diminished at longer time points (Figure 6A). The signal

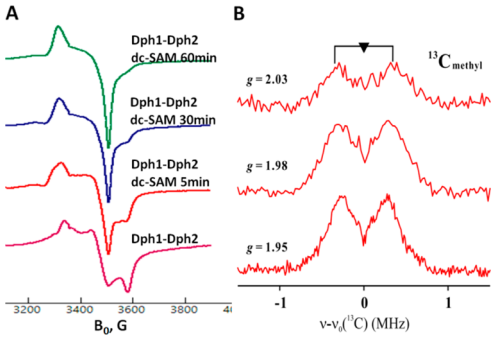

Figure 6. (A) X-band CW EPR spectra of Dph1-Dph2 in the absence and presence of dc-SAM at 12 K. (B) 2D field-frequency 35 GHz ^{13}C Mims ENDOR of the 60 min sample of Dph1-Dph2 with $^{13}C_{methyl}$-dc-SAM. Experimental fields are as indicated. Conditions: MW frequency = 34.8 GHz, MW pulse length ($\pi/2$) = 50 ns, τ = 500 ns, and T = 2 K.

broadened beyond detection at 35 K (Figure S7). The *g*-values and temperature dependence of the new species indicated it involved the cluster, and was not an organic radical. This new species dissappeared when we added DTT, which indicated it was a reactive intermediate (Figure S8).

We further characterized this intermediate by preparing it with $^{13}C_{methyl}$-dc-SAM and using Mims pulsed electron-nuclear double resonance (ENDOR) spectroscopy. A small, apparently near-isotropic coupling, $a_{iso} \approx 0.6$ MHz, was detected (Figure 6B). Such a small coupling indicated the ^{13}C-methyl group was in the vicinity of the cluster but weakly interacting with its spin. In previous studies of pyruvate formate lyase-activate enzyme (PFL-AE), the nonlocal through-space coupling constant is 0.33 MHz for ^{13}C-Me SAM,[16] and the isotropic hyperfine coupling constant of ^{13}C-carboxyl SAM is 0.71 MHz.[13] The methyl group could have been transferred to a molecule adjacent, but we propose it bound to a sulfur atom of the [4Fe-4S] cluster. Without precedent, the spin density on such a sulfur would be small, and would plausibly lead to the small observed coupling for a cluster S-methyl. Such a species could arise if, instead of the reduced cluster causing homolytic cleavage with the natural substrate SAM, a S_N2 displacement mechanism occurred: a sulfur atom in the reduced [4Fe-4S] cluster attacked the sulfonium center of dc-SAM, forming a S-methyl-[4Fe-4S] intermediate. Other nucleophiles, such as the thiol groups on DTT or nucleophilic residues on proteins, would subsequently attack the S-methyl-[4Fe-4S] intermediate and complete the methyl transfer reaction (Figure 7). This

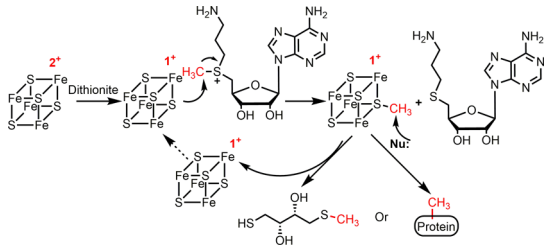

Figure 7. Mechanism of *Ph*Dph2 catalyzed methyl transfer with dc-SAM.

intermediate could be compared to the catalytic organometallic intermediate in PFL-AE.[17] Both intermediates are formed by substrate (SAM or SAM analogue) cleavage with bonding of nascent radical/cleavage product to [4Fe-4S] cluster, with the difference being whether the Fe or the S atom in the cluster is directly bonded. As a test of this mechanism, we reasoned this intermediate should produce methanethiol after denaturation of the protein and Fe-S cluster in acidic conditions. Indeed, we detected the formation of methanethiol after denaturation of the protein subjected to turnover, whereas no methanethiol was detected in the control reaction without dithionite (Figure S9).

Computational studies suggest the bidentate coordination of the amino and carboxyl groups of SAM to the unique cluster iron of conventional radical SAM enzymes is essential for the generation of the 5′-dA radical. First, the sulfonium ion and unique iron orbitals should have matching energies, required for electron transfer between two atoms.[18] Second, the bidentate structure and the resulting conformation of SAM explain the regioselectivity of $C_{5'}$, $_{Ade}$-S bond cleavage.[19] *Ph*Dph2, representing a different type of radical SAM enzymes, cleaves the $C_{\gamma,Met}$-S bond in SAM and produces an ACP radical. Our results indicate *Ph*Dph2-catalyzed reaction also requires both the amino group and carboxylate group of SAM. However, unlike classical radical SAM enzymes, *Ph*Dph2 can still accept the dc-SAM analogue as a substrate but catalyzes the cleavage of the C_{methyl}-S bond and not the $C_{\gamma,Met}$-S bond cleaved in the natural enzymatic reaction. Instead of a CxxxCxxC motif in traditional radical SAM enzyme, the three conserved cysteine residues of *Ph*Dph2 that bind the [4Fe-4S] cluster are located in separate structural domains separated by more than 100 residues in the primary sequence.[7] We postulate this structure allows SAM to bind the [4Fe-4S] in *Ph*Dph2 different from that in classical radical SAM enzymes. The different binding mode of SAM in *Ph*Dph2 (and yeast Dph1-Dph2) likely contributes to the stereoelectronic control of SAM cleavage and the unique reactivity with the SAM analogues described.

Although we cannot rule out the possibility of a radical mechanism for formation of the S-methyl-[4Fe-4S] intermediate, the S_N2 nucleaphilic mechanism is most likely for the following reasons: (1) A homolytic cleavage of the C_{methyl}-S bond in SAM is

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difficult due to the high energy of methyl radical;[4] (2) If a methyl radical is generated, it should recombine with the closest unique iron and form a C-Fe bond, as the organometallic intermediate in PFL-AE.[17] Although the formation of low level of 5′-deoxy-5′-thioadenosine, a reaction product of 5′-deoxyadenosyl radical with one sulfur in the oxidized cluster, was reported in NosL study, the iron sulfur cluster was inactivated instead of playing a catalytic role.[20] We therefore postulate this is the first case in which a [4Fe-4S]$^{+}$ cluster in a radical SAM enzyme switches from catalyzing an Fe-based one electron transfer reaction to catalyzing a S-based two electron transfer reaction by changing the structure of SAM. This activity of *Ph*Dph2 is dependent on the reduced cluster [4Fe-4S]$^{+}$, which is directly involved in the methyl transfer reaction.

The methyl transfer studied is different from that in traditional methyltransferase and other radical SAM enzymes with methyl transfer activity. The methylsynthases RimN and Cfr[21−23] use conserved cysteine residues to cleave the methyl group from SAM via an S_N2 mechanism. RimO and MiaB,[24−26] representing a subgroup of methylthio transferase in radical SAM enzyme family, consume 2 molecules SAM: one donates a methyl group to the external sulfur attached to 4Fe-4S cluster, another SAM is reductively cleaved to generate a 5′-dA radical, which abstracts a proton from the substrate to generate a substrate radical. The substrate radical in turn attacks the methylthio group on the 4Fe-4S cluster to complete methylthio transfer. In our case, *Ph*Dph2 or yeast Dph1-Dph2 only consumes 1 molecule SAM analogue in the catalytic cycle and the methyl acceptor is a sulfur of the [4Fe-4S] cluster. A study reported NosN can use MTA, instead of SAM, as a direct methyl donor.[27] The methyltransferase activity of *Ph*Dph2 on dc-SAM resembles the cobalamin dependent S_N2-based methyltransferase.[28] Instead of the reduced cobalt in methyl-cobalamin, the sulfur in [4Fe-4S]$^{+}$ serves as an intermediate methyl acceptor to transfer methyl group to other nucleophiles. Our studies have revealed the [4Fe-4S] cluster in a radical SAM enzyme can be tuned to cleave any one of the three bonds to the sulfonium sulfur of SAM or analogues. Together with studies on PFL-AE and *Ph*Dph2,[17,29] we provide evidence for the versatility of these [4Fe-4S] clusters.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b01712.

Materials and methods, supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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