

# Dph3 Enables Aerobic Diphthamide Biosynthesis by Donating One Iron Atom to Transform a [3Fe–4S] to a [4Fe–4S] Cluster in Dph1–Dph2

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**ABSTRACT:** All radical *S*-adenosylmethionine (radical-SAM) enzymes, including the noncanonical radical-SAM enzyme diphthamide biosynthetic enzyme Dph1–Dph2, require at least one [4Fe–4S](Cys)<sub>3</sub> cluster for activity. It is well-known in the radical-SAM enzyme community that the [4Fe–4S](Cys)<sub>3</sub> cluster is extremely air-sensitive and requires strict anaerobic conditions to reconstitute activity *in vitro*. Thus, how such enzymes function *in vivo* in the presence of oxygen in aerobic organisms is an interesting question. Working on yeast Dph1–Dph2, we found that consistent with the known oxygen sensitivity, the [4Fe–4S] cluster is easily degraded into a [3Fe–4S] cluster. Remarkably, the small iron-containing protein Dph3 donates one Fe atom to convert the [3Fe–4S] cluster in Dph1–Dph2 to a functional [4Fe–4S] cluster during the radical-SAM enzyme catalytic cycle. This mechanism to maintain radical-SAM enzyme activity in aerobic environments is likely general, and Dph3-like proteins may exist to keep other radical-SAM enzymes functional in aerobic environments.

Radical *S*-adenosylmethionine (radical-SAM) enzymes are found in all domains of life.<sup>1</sup> They utilize a [4Fe–4S] cluster and SAM to initiate a diverse set of radical reactions.<sup>2</sup> In the active site, radical-SAM enzymes contain a [4Fe–4S] cluster in which three of the four iron atoms are coordinated by cysteines and the fourth iron is coordinated by SAM.<sup>2</sup> In the absence of SAM, the fourth iron in the [4Fe–4S] cluster is not coordinated, rendering it prone to oxidation.<sup>2</sup> In most cases, a brief exposure of a radical-SAM enzyme to air leads to the formation of an inactive [3Fe–4S] cluster through the loss of the fourth iron.<sup>2,3</sup> The instability of the [4Fe–4S] cluster in air leads to the question how such an unstable cluster could function well in aerobes.<sup>3</sup>

The Dph1–Dph2 heterodimer is a noncanonical radical-SAM enzyme in eukaryotes that is essential for the first step of biosynthesis of diphthamide.<sup>4,5</sup> Diphthamide is a unique post-translationally modified histidine residue on eukaryotic elongation factor 2 (eEF2), a GTPase that is essential in the elongation step of translation.<sup>6–10</sup> The diphthamide biosynthesis in eukaryotes requires four steps involving at least seven proteins.<sup>9,11</sup> The Dph1–Dph2 heterodimer catalyzes the addition of an aminocarboxypropyl (ACP) group to a specific histidine residue in eEF2 using SAM as a substrate (Figure 1A,B).<sup>5</sup> During the *in vitro* enzymatic reaction, the [4Fe–4S]<sup>2+</sup> cluster in Dph1–Dph2 is reduced to [4Fe–4S]<sup>1+</sup> using dithionite as the reductant.<sup>12</sup> The [4Fe–4S]<sup>1+</sup> cluster donates two electrons to SAM, cleaving it, forming an organometallic complex (intermediate I) and releasing methylthioadenosine (Figure 1A).<sup>13</sup> The organometallic intermediate serves as a stabilized ACP radical and reacts with eEF2 to form intermediate II, which is converted to the ACP-modified eEF2 product after loss of a hydrogen atom (Figure 1A).<sup>10,13</sup>

Under physiological conditions, the reduction of the [4Fe–4S] clusters in Dph1–Dph2 requires Dph3 and Cbr1.<sup>14,15</sup> Dph3 contains a zinc finger domain that binds one iron with four cysteine residues.<sup>16</sup> The iron in Dph3 is redox-active and can be reduced by FAD-bound Cbr1 with NADH in baker's yeast.<sup>14,15</sup>

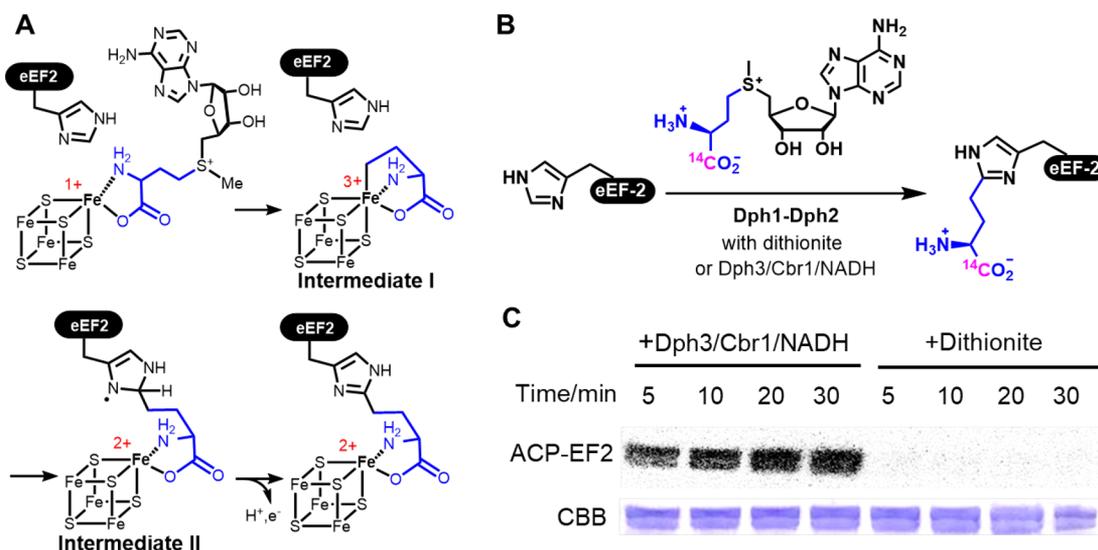
In yeast and other aerobic eukaryotes, Dph1–Dph2, Dph3, and Cbr1 reside in the cytosol, a hostile environment for oxygen-sensitive radical-SAM enzymes.<sup>17</sup> How the first step in diphthamide biosynthesis happens in yeast in the presence of oxygen is an interesting question. Although Dph3 and Cbr1 may be able to reduce the [4Fe–4S] cluster in Dph1–Dph2, maintaining the [4Fe–4S] cluster in Dph1–Dph2 would be challenging given that *in vitro* the [4Fe–4S] cluster is quickly degraded to [3Fe–4S], a common property for all radical-SAM enzyme Fe–S clusters. Here we propose a model in which Dph1–Dph2 maintains a [3Fe–4S] cluster and Dph3 provides the fourth Fe during the enzymatic reaction to form the active [4Fe–4S] cluster *in situ* to catalyze the reaction. This strategy nicely solves the oxygen-sensitivity problem of Dph1–Dph2 radical-SAM enzyme and may be generally used to allow radical-SAM enzymes to function in aerobic environments.

In an attempt to reconstitute the first step of diphthamide biosynthesis, we found that an old batch of anaerobically

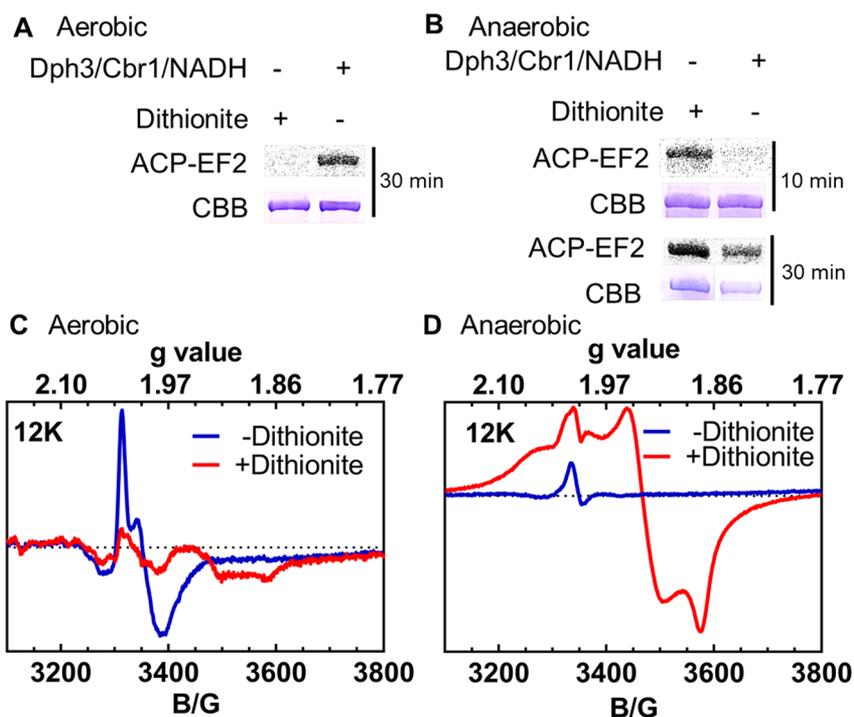
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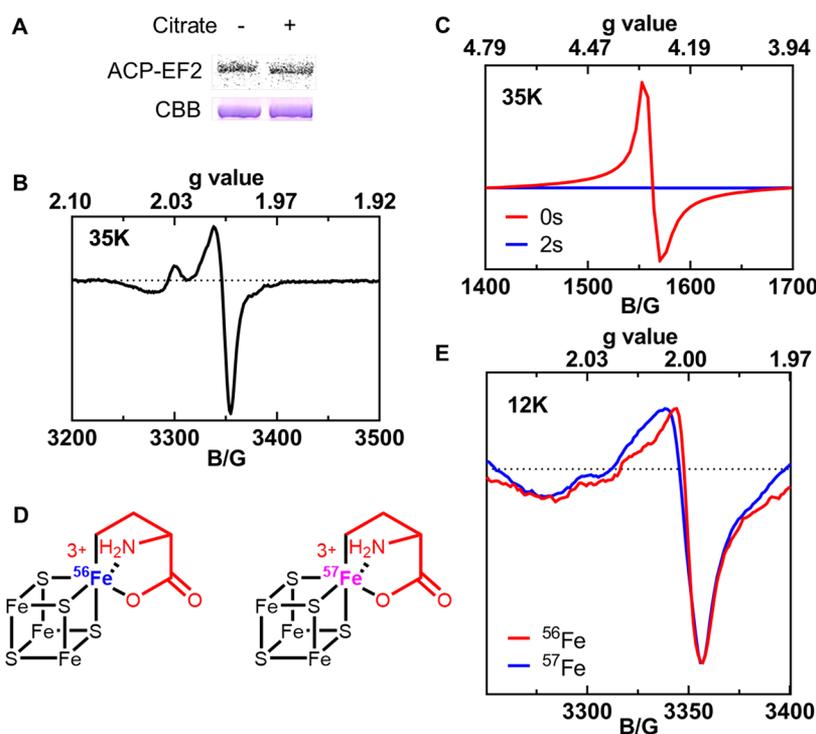
**Figure 1.** An old batch of Dph1–Dph2 required Dph3/Cbr1/NADH but not dithionite for activity. (A) Reaction mechanism for the first step of diphthamide biosynthesis. Intermediate I is organometallic in nature. (B) Scheme showing the *in vitro* reconstitution of the Dph1–Dph2 enzymatic reaction. Dph1–Dph2 catalyzes the addition of the ACP group to histidine using SAM as the substrate. eEF2 incorporates  $^{14}\text{C}$ -modified ACP when carboxy- $^{14}\text{C}$ -SAM is used as the substrate. (C) *In vitro* reconstitution of the first step of diphthamide biosynthesis on eEF2 with Dph1–Dph2 and carboxy- $^{14}\text{C}$ -SAM using Dph3/Cbr1/NADH (left four lanes) or dithionite (right four lanes) as the reducing agent: (top) autoradiography showing labeled eEF2 product; (bottom) eEF2 stained with Coomassie blue (CBB).



**Figure 2.** Dph3 activates aerobically purified Dph1–Dph2. (A) *In vitro* reconstitution of the first step of diphthamide biosynthesis with aerobically purified Dph1–Dph2. The reaction was carried out aerobically. The reaction time is labeled on the right. (B) *In vitro* reconstitution of the first step of diphthamide biosynthesis with anaerobically purified Dph1–Dph2. The reaction was carried out anaerobically. The reaction time is labeled on the right. For the reaction with dithionite, Dph1–Dph2 was mixed with eEF2 and carboxy- $^{14}\text{C}$ -SAM, and dithionite was added to initiate the reaction. For the reaction with Dph3/Cbr1/NADH, Dph1–Dph2 was mixed with Dph3, Cbr1, eEF2, and carboxy- $^{14}\text{C}$ -SAM. NADH was added to initiate the reaction. (top) Autoradiography showing labeled eEF2 product. (bottom) eEF2 stained with Coomassie blue (CBB). (C) X-band continuous wave (CW) EPR signal of aerobically purified Dph1–Dph2 with or without dithionite obtained at 12 K. (D) X-band CW EPR signal of anaerobically purified Dph1–Dph2 with or without dithionite obtained at 12 K. The quantification of Fe–S cluster species is included in Supplementary Table 1.

purified Dph1–Dph2 functions only with the physiological reducing system,<sup>15</sup> Dph3/Cbr1/NADH, and not with the small-molecule reductant dithionite (Figure 1C).

Dithionite is generally used for *in vitro* reconstitution of radical-SAM enzymes. Dithionite reduces  $[\text{4Fe–4S}]^{2+}$  into  $[\text{4Fe–4S}]^{1+}$ , thus forming the active Fe–S cluster. Activation



**Figure 3.** Dph3 provides the unique Fe to complete the [4Fe-4S] cluster during the reaction. (A) The activity of aerobically purified Dph1–Dph2 with Dph3 is not affected by citrate, as shown by *in vitro* reconstitution of the first step of diphthamide biosynthesis on eEF2 using aerobically purified Dph1–Dph2, carboxy- $^{14}\text{C}$ -SAM, Cbr1, NADH and Dph3 in the presence or absence of 2 mM citrate: (top) autoradiography showing labeled eEF2 product; (bottom) eEF2 stained with Coomassie blue (CBB). (B) Dph1–Dph2, Dph3, eEF2, Cbr1, and NADH were mixed at room temperature and subjected to rapid freeze quench (RFQ) at 2 s. The quenched reaction mixture was monitored using X-band CW EPR spectroscopy at 35 K in the region of 3100 to 3500 G. (C) The quenched reaction mixture (2 s) was monitored using X-band CW EPR spectroscopy at 12 K in the region of 1400 to 1700 G to detect the Dph3 Fe signal. The spectrum was compared to the reaction mixture before addition of NADH (0 s). (D) Intermediate I structure with (left)  $^{56}\text{Fe}$  or (right)  $^{57}\text{Fe}$  at the unique iron position. (E) Dph1–Dph2 (with  $^{56}\text{Fe}$ ),  $^{57}\text{Fe}$ -Dph3, eEF2, Cbr1, SAM, and NADH were mixed at room temperature and subjected to RFQ at 2 s. The quenched reaction mixture was monitored using X-band CW EPR spectroscopy at 12 K. The EPR signal was aligned to that obtained with  $^{56}\text{Fe}$ -Dph3. A 5.9 G line broadening was observed with  $^{57}\text{Fe}$ -Dph3, indicating the incorporation of  $^{57}\text{Fe}$  from Dph3 into the intermediate I signal.

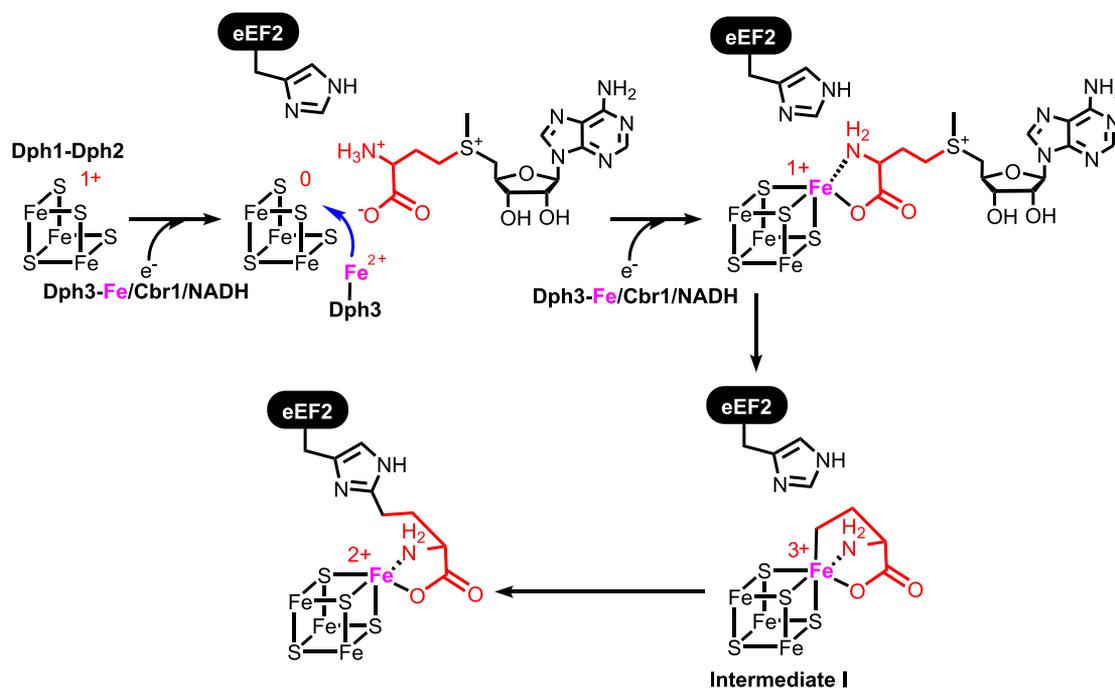
of both Dph1–Dph2 and the archaeal homologue PhDph2 by dithionite was achieved experimentally previously.<sup>4,5</sup> The observation that this particular batch of Dph1–Dph2 could be activated with Dph3/Cbr1/NADH but not with dithionite led us to hypothesize that the [4Fe-4S] cluster in Dph1–Dph2 was decomposed to the [3Fe-4S] cluster as a result of accidental exposure to oxygen, rendering dithionite unable to activate it. However, Dph3/Cbr1/NADH could provide the fourth Fe to reconstitute the active [4Fe-4S] cluster.

To test the hypothesis, we purified a batch of Dph1–Dph2 aerobically. The aerobically purified Dph1–Dph2 showed little activity with dithionite but showed much higher activity with Dph3/Cbr1/NADH (Figure 2A). In contrast, Dph1–Dph2 purified anaerobically showed higher activity with dithionite than with Dph3/Cbr1/NADH (Figure 2B). In line with the *in vitro* reconstitution results, electron paramagnetic resonance (EPR) spectroscopy showed that aerobically purified Dph1–Dph2 contains mostly [3Fe-4S]<sup>1+</sup> and after dithionite reduction produces very little [4Fe-4S]<sup>1+</sup> EPR signal (Figure 2C). In contrast, the anaerobically purified Dph1–Dph2 contained very little [3Fe-4S]<sup>1+</sup> signal and produced a strong [4Fe-4S]<sup>1+</sup> signal after dithionite reduction (Figure 2D). For the anaerobically purified Dph1–Dph2 containing mostly the [4Fe-4S]<sup>2+</sup> cluster, dithionite can better reduce the [4Fe-4S]<sup>2+</sup> cluster because of its lower reduction potential compared with Dph3/Cbr1/NADH, explaining why the reaction worked

better with dithionite in the case of anaerobically purified Dph1–Dph2.

The above data confirmed that the aerobically purified Dph1–Dph2 containing mostly [3Fe-4S]<sup>1+</sup> shows high activity with Dph3/Cbr1/NADH but little to no activity with dithionite. Thus, the Dph3/Cbr1/NADH system is able to provide the fourth Fe to reconstitute the active [4Fe-4S] cluster needed for the enzymatic activity. The next question concerns the origin of the fourth Fe. Because Dph3 binds one Fe ion using four cysteine residues, we reasoned that this Dph3-bound Fe could be transferred to Dph1–Dph2 to reconstitute the [4Fe-4S] cluster from the [3Fe-4S] cluster. The possibility that the fourth iron comes from adventitious Fe ions in the solution is unlikely, as the dithionite-reduced aerobically purified Dph1–Dph2 could not form the [4Fe-4S] cluster (Figure 2B). To further rule out the possibility that the fourth Fe is from adventitious Fe in the solution, we used citrate to limit the free iron in the solution.<sup>18,19</sup> The activity of aerobically purified Dph1–Dph2 with Dph3/Cbr1/NADH remained intact supporting the hypothesis that the fourth Fe is not from adventitious iron in the reaction mixture (Figure 3A).

To further confirm that Dph3 provides the fourth Fe to reconstitute the [4Fe-4S] cluster in Dph1–Dph2, we sought to detect the [4Fe-4S]<sup>1+</sup> cluster signal by EPR spectroscopy. Interestingly, in the solution with Dph1–Dph2 and Dph3/Cbr1/NADH but without the substrates (eEF2 and SAM), we



**Figure 4.** Proposed mechanism for the Dph1–Dph2 reaction with Dph3 donating the fourth Fe for the [4Fe–4S] cluster. The aerobically purified Dph1–Dph2 contains a [3Fe–4S]<sup>1+</sup> cluster. The [3Fe–4S]<sup>1+</sup> cluster is reduced to [3Fe–4S]<sup>0</sup> in the presence of Dph3/Cbr1/NADH. When both eEF2 and SAM are present, Fe<sup>2+</sup> from Dph3 is transferred to Dph1–Dph2, forming the [4Fe–4S]<sup>2+</sup> cluster, which at the same time is reduced by Dph3/Cbr1/NADH. The [4Fe–4S]<sup>1+</sup> cluster then forms intermediate I, which eventually leads to the final product, ACP-modified eEF2.

could not detect the [4Fe–4S]<sup>1+</sup> EPR signal (data not shown). We hypothesized that the [4Fe–4S]<sup>1+</sup> cluster is formed only when both of the substrates (eEF2 and SAM) are present, which then quickly react to form products. Thus, to detect any potential [4Fe–4S] cluster EPR signal during the reaction, we performed rapid freeze quench (RFQ). Previously, two intermediates, organometallic intermediate I and organic radical intermediate II, were captured and characterized (Figure 1A).<sup>13</sup> If our hypothesis that Dph3 provides the fourth iron to reconstitute the active [4Fe–4S] cluster in Dph1–Dph2 is true, we should be able to capture and detect either the [4Fe–4S]<sup>1+</sup> signal or the intermediate I signal by RFQ. Aerobically purified Dph1–Dph2 was mixed with Dph3, eEF2, SAM, and a catalytic amount of Cbr1. The reaction was triggered by mixing the protein mixture with NADH and then quenched at 2 s, and the reaction mixture was analyzed by EPR spectroscopy. The intermediate I signal was successfully detected (Figure 3B), indicating that the [4Fe–4S]<sup>1+</sup> cluster was formed during the reaction and then quickly converted to intermediate I<sup>13</sup> (Figure 4). At the same time, there was a fast decrease in the Fe<sup>3+</sup> signal in Dph3 (Figure 3C). The disappearance of the Fe<sup>3+</sup> signal is mostly due to reduction to Fe<sup>2+</sup> in Dph3 right after NADH addition.

To further confirm that Dph3 is the source of the unique iron in intermediate I, we obtained <sup>57</sup>Fe-labeled Dph3. If the <sup>57</sup>Fe is transferred during the reaction, we should observe line broadening of the intermediate I signal.<sup>13</sup> We performed the same RFQ experiment with <sup>57</sup>Fe-labeled Dph3, and indeed, we saw a 5.9 G line broadening of the intermediate I signal (Figure 3D,E). The line broadening effect is similar to what we observed previously<sup>13</sup> and falls within the range of <sup>57</sup>Fe hyperfine coupling.<sup>20,21</sup> This result further supports the conclusion that the unique iron essential for the reaction is obtained from Dph3 during the reaction.

Thus, Dph3 enables the radical-SAM enzyme Dph1–Dph2 to function aerobically. Dph1–Dph2 in the resting state exists in [3Fe–4S] form. During the reaction, Dph3 transfers an iron to Dph1–Dph2 to reconstitute the [4Fe–4S] cluster in situ. At the same time, Dph3/Cbr1/NADH provides an electron to reduce the 4Fe–4S cluster.<sup>5</sup> The reformed [4Fe–4S]<sup>1+</sup> cluster cleaves SAM and forms intermediate I (Figure 4).

When Dph1–Dph2 is purified anaerobically, Dph1–Dph2 contains the full [4Fe–4S]<sup>2+</sup> cluster.<sup>5</sup> Dph3 mediated the electron transfer to reduce [4Fe–4S]<sup>1+</sup> and trigger the reaction.<sup>5</sup> When Dph1–Dph2 is purified aerobically, Dph1–Dph2 contains the [3Fe–4S]<sup>1+</sup> cluster. In this system, Dph3 has two functions: it mediates the electron transfer and transfers Fe to reform the active [4Fe–4S]<sup>1+</sup> cluster to trigger the reaction. Our current model reveals the additional function of Dph3 to complete the [4Fe–4S] cluster along with its ability to mediate the electron transfer. The detailed mechanism of electron transfer mediated by Dph3 is still under investigation.

Our model reveals an elegant solution that nature uses to allow Dph1–Dph2 radical-SAM enzymes to function aerobically. The [4Fe–4S] cluster is not stable in the presence of oxygen, while the [3Fe–4S] cluster in Dph1–Dph2 and the Fe bound by Dph3 are both stable. Furthermore, the reconstitution of the active [4Fe–4S] cluster occurs only when both substrates (eEF2 and SAM) are present, which allows maximum efficiency of the system (if the active cluster were formed when eEF2 or SAM is not present, it would be quickly destroyed by oxygen and thus wasted). This is likely a general solution that aerobes use to cope with the oxygen-sensitivity problem of radical-SAM enzymes, allowing oxygen-sensitive Fe–S enzymes to function in an aerobic environment. In yeast and humans, there are other radical-SAM enzymes that function in the cytosol,<sup>22</sup> including tRNA<sup>tyrosyl</sup>-

synthesizing protein (Tyw1),<sup>23</sup> the elongator complex,<sup>24,25</sup> viperin,<sup>26–28</sup> and molybdenum cofactor biosynthesis protein 1 (MOCS1).<sup>29</sup> These radical-SAM enzymes are also oxygen-sensitive, and thus, they may also require proteins like Dph3 to help to keep them active in an aerobic environment.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.1c03956>.

Materials and methods and supplementary figures and tables (PDF)

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### Notes

The authors declare no competing financial interest.

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