JBIC Journal of Biological Inorganic Chemistry (2019) 24:777–782
https://doi.org/10.1007/s00775-019-01702-0

ORIGINAL PAPER

The asymmetric function of Dph1–Dph2 heterodimer in diphthamide biosynthesis

Min Dong[1,2] · Emily E. Dando[1] · Ilana Kotliar[1] · Xiaoyang Su[1] · Boris Dzikovski[1] · Jack H. Freed[1] · Hening Lin[1,3]

Received: 18 June 2019 / Accepted: 6 August 2019 / Published online: 28 August 2019

Abstract

Diphthamide, the target of diphtheria toxin, is a post-translationally modified histidine residue found in archaeal and eukaryotic translation elongation factor 2 (EF2). In the first step of diphthamide biosynthesis, a [4Fe–4S] cluster-containing radical SAM enzyme, Dph1–Dph2 heterodimer in eukaryotes or Dph2 homodimer in archaea, cleaves *S*-adenosylmethionine and transfers the 3-amino-3-carboxypropyl group to EF2. It was demonstrated previously that for the archaeal Dph2 homodimer, only one [4Fe–4S] cluster is necessary for the in vitro activity. Here, we demonstrate that for the eukaryotic Dph1–Dph2 heterodimer, the [4Fe–4S] cluster-binding cysteine residues in each subunit are required for diphthamide biosynthesis to occur in vivo. Furthermore, our in vitro reconstitution experiments with Dph1–Dph2 mutants suggested that the Dph1 cluster serves a catalytic role, while the Dph2 cluster facilitates the reduction of the Dph1 cluster by the physiological reducing system Dph3/Cbr1/NADH. Our results reveal the asymmetric functional roles of the Dph1–Dph2 heterodimer and may help to understand how the Fe–S clusters in radical SAM enzymes are reduced in biology.

Keywords Diphthamide biosynthesis · Radical SAM enzyme · Iron–sulfur cluster

Introduction

Diphthamide is a post-translationally modified residue on archaeal and eukaryotic elongation factor 2 (EF2). It is so named because it is the target of diphtheria toxin, which specifically recognizes and ADP-ribosylates it to inactivate EF2 and inhibit host cell protein synthesis [1, 2]. Diphthamide has captured the attention of many researchers in the past few decades due to its interesting chemistry and biology [3–6]. The biosynthesis of diphthamide, for example, has been fascinating and significant advances in the enzymology of diphthamide biosynthesis have been made in the past decade. Diphthamide biosynthesis occurs in four steps (Fig. 1) [4, 7]. In the first step, a 3-amino-3-carboxypropyl (ACP) group is transferred from *S*-adenosylmethionine (SAM) to the target histidine residue on EF2, forming a C–C bond. The second step requires protein Dph5, a methyltransferase that methylates the amino and carboxylate of the ACP group. The third step is the hydrolysis of the methyl ester catalyzed by protein Dph7 [8, 9]. The last step is the amidation of the carboxylate group of diphthine catalyzed by Dph6 [10, 11].

The first step of diphthamide biosynthesis is the most interesting step from the enzymology perspective. There are at least four proteins (Dph1-Dph4) required for this step in eukaryotes [7]. Dph1–Dph2 heterodimer is a non-canonical radical SAM enzyme that transfers the ACP group from SAM to EF2 [12]. In archaea, which only contain Dph2, the Dph2 homodimer is the radical SAM enzyme responsible for the first step [13]. Eukaryotic Dph1–Dph2 heterodimer or archaeal Dph2 homodimer both contain [4Fe–4S] clusters, similar to canonical radical SAM enzymes. The [4Fe–4S] clusters are each coordinated with three cysteine residues and the unique Fe that is not coordinated by protein cysteine residues binds SAM [13]. In the reduced state, the [4Fe–4S] cluster provides one electron to reductively cleave SAM. Recent studies showed that this cleavage leads to the formation of an organometallic intermediate with an iron–carbon (Fe–C) bond between ACP and the enzyme's [4Fe–4S]

✉ Hening Lin
hl379@cornell.edu

[1] Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA

[2] Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

[3] Howard Hughes Medical Institute, Cornell University, Ithaca, NY 14853, USA

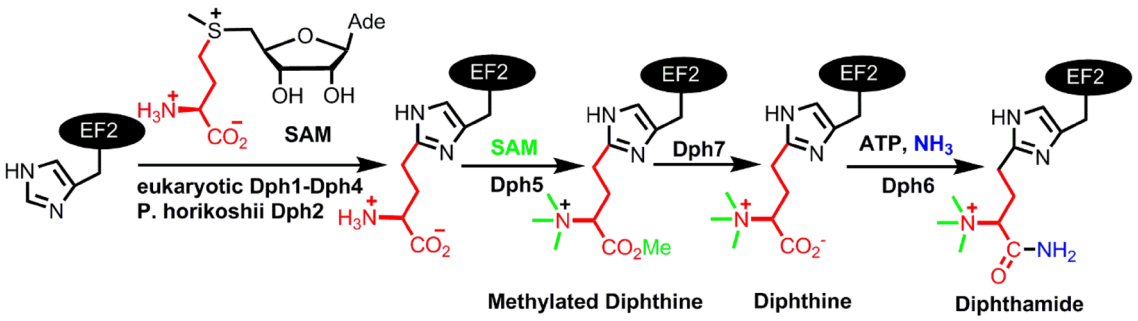

Fig. 1 Diphthamide biosynthetic pathway

cluster [14]. In the presence of the substrate protein EF2, the homolysis of the Fe–C bond generates an ACP radical to add to the histidine side chain. Previously, it was also shown that in the *Pyrococcus horikoshii* Dph2 (*Ph*Dph2) homodimer, when the cysteine residues coordinating the [4Fe–4S] clusters in one monomer were mutated, the resulting Dph2 homodimer was still active [15]. This suggests that only one [4Fe–4S] cluster is required for the activity of PhDph2 homodimer. Given this knowledge, it is interesting to consider the eukaryotic Dph1–Dph2 heterodimer. Do Dph1 and Dph2 each bind a [4Fe–4S] cluster? If so, what is the role of each [4Fe–4S] cluster?

In the present study, we find that the cysteine residues that supposedly coordinate the [4Fe–4S] clusters in eukaryotic Dph1 and Dph2 are important for Dph1–Dph2 activity in vivo, suggesting that Dph1 and Dph2 likely each bind a [4Fe–4S] cluster. Interestingly, in vitro when the cysteine residues in Dph1 were mutated, no catalytic activity was observed, but when cysteine residues in Dph2 were mutated, the Dph1–Dph2 heterodimer was still active when dithionite was used as the reductant. However, when the natural reduction system, Dph3/Cbr1/NADH, was used as the reductant, Dph1–Dph2 was not active anymore when the cysteine residues in Dph2 were mutated. Our data thus support a model in which the Dph1 Fe–S cluster serves a catalytic role, while the Dph2 Fe–S cluster facilitates the reduction of Dph1 cluster by the Dph3/Cbr1/NADH system.

Materials and methods

Protein expression and purification

S. cerevisiae Dph1–Dph2 heterodimer wide type was expressed and purified as previously reported [14] and stored in 150 mM NaCl, 1 mM DTT and 200 mM Tris–HCl at pH 7.4 and 5% glycerol. Iron and sulfur contents of the protein were analyzed as previously reported [14]. The as-isolated Dph1–Dph2 heterodimer usually has 30–50% cluster loading (assuming that each unit of Dph1 and Dph2 binds a 4Fe–4S cluster based on the structure of the *Ph*Dph2 homodimer).

Dph1–Dph2 mutants were generated by site-directed mutagenesis. The corresponding mutant proteins were prepared as the Dph1–Dph2 wide type.

Cloning, expression and purification of *S. cerevisiae* Dph3, Cbr1 and EF2

S. cerevisiae Dph3, EF2 and truncated Cbr1 were prepared as previously reported [14]. Aerobically purified proteins were degassed by Schlenk line before use.

EPR spectroscopy

X band EPR spectra were recorded on a Bruker ElexSys E500 EPR spectrometer at a frequency of 9.38 GHz. EPR measurements at 12 K were carried out using an ESR 910 liquid-helium cryostat (Oxford Instruments). The spectrometer settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 8 G; microwave power, 0.63 mW. The field sweeps were calibrated with a Bruker ER 035 Gauss meter and the microwave frequency was monitored with a frequency counter. Data acquisition and manipulation were performed with Xepr software. Dph1M–Dph2 (496 µM) or Dph1–Dph2M (458 µM), with a volume of 50 µl reduced by dithionite (final 10 mM) in the anaerobic chamber. The solution was incubated for 1 min and transferred to EPR tubes and frozen in liquid N2. Then the tubes were sealed in the anaerobic chamber and taken out for EPR analysis.

In vivo diphtheria toxin resistance yeast growth assay

Single or double mutants of Dph1 and Dph2 were made by overlap extension PCR. Δ*dph1* or Δ*dph2* strains were transformed with the various *dph1* or *dph2* encoding plasmids, together with the pLMY101 plasmid which encodes the diphtheria toxin using the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA). Transformed yeast cells were

grown on synthetic complete medium with histidine and uracil dropout with 2% glucose as the carbon source. For the survival assay, 2% galactose was used as the carbon source. Colony formation was recorded 3 days after plating.

Anaerobic reconstitution of Dph1–Dph2 activity

The reactions contained 20 µM Dph1–Dph2 wt or mutants, 10 µM of Dph3, 5 µM of Cbr1, 200 µM of NADH and 7 µM of eEF2 in the buffer of 150 mM NaCl, 1 mM DTT and 200 mM Tris–HCl at pH 7.4. The reaction mixtures were assembled in the anaerobic chamber under strictly anaerobic conditions. The reaction vials were sealed before being taken out of the anaerobic chamber. ^{14}C-SAM (final concentration of 18 µM) was injected into each reaction vial with a microliter syringe to start the reaction. The reaction mixtures were vortexed briefly to mix and incubated at 30 °C for 1 h. The reactions were stopped by adding protein loading dye to the reaction mixture and subsequently heating at 95 °C for 5 min, followed by 12% SDS–polyacrylamide gel electrophoresis. The dried gel was exposed to a PhosphorImaging screen (GE Healthcare) and the radioactivity was detected using a STORM 860 PhosphorImager (GE Healthcare).

Results and discussion

Dph1–Dph2 complex contains [4Fe–4S] clusters

In archaea, *Ph*Dph2 catalyzes the first step of diphthamide biosynthesis. *Ph*Dph2 is a homodimer, and each monomer has three conserved cysteine residues that bind a [4Fe–4S] cluster [13]. Yeast Dph1 and Dph2 are both orthologous to *Ph*Dph2 and form a heterodimer. Thus, we wanted to characterize whether yeast Dph1–Dph2 heterodimer also binds to two [4Fe–4S] clusters. Consistent with previous reports on *Ph*Dph2, the anaerobically purified Dph1–Dph2 complex shows a broad absorption at 410 nm, typical of [4Fe–4S] cluster, which disappears on reduction by dithionite [13, 16]. The EPR spectrum of Dph1–Dph2 also shows $[4Fe–4S]^{+}$ signal [16]. Quantification of the Fe and S contents of the anaerobically purified yeast Dph1–Dph2 heterodimer showed that the heterodimer contains 4.8 equivalent of iron and 4.4 equivalent of sulfur. Therefore, these results imply that the Dph1–Dph2 complex may contain two [4Fe–4S] clusters, but due to the low iron and sulfur contents, we could not rule out other possibilities at this time.

Potential [4Fe–4S]-binding cysteine residues in both Dph1 and Dph2 are necessary for the in vivo activity

To further confirm that Dph1 and Dph2 each binds an Fe–S cluster, we next decided to carry out mutagenesis studies of key Fe–S cluster-binding residues. Sequence alignment shows that *Ph*Dph2 is more similar to yeast Dph1 than to yeast Dph2 (Fig. 2). There are three cysteine residues in *Ph*Dph2 that coordinate the [4Fe–4S] cluster, Cys59, Cys163 and Cys287 (Fig. 2, red *). The corresponding residues are Cys133, Cys239 and Cys368 in yeast Dph1. In contrast, in yeast Dph2, Cys106 and Cys362 correspond to *Ph*Dph2 Cys59 and Cys287, and there is no cysteine that corresponds to *Ph*Dph2 Cys163. We decided to find out whether mutation of these potential Fe–S cluster coordinating residues would disrupt diphthamide biosynthesis. If the conserved Cys residues indeed bind the Fe–S cluster, we would expect that mutating any of them would eliminate the catalytic activity.

To test this, we mutated the three corresponding Cys residues (C133, C239, and C368) in yeast Dph1 to Ala and transformed the three mutants into the Δ*dph1* strain separately that contains pLMY101 plasmid encoding diphtheria toxin (DT) under control of a GAL1 promoter. On plates containing galactose medium that induces DT expression, cells containing the three mutants or empty vector could grow (insensitive to DT, no diphthamide formation), while cells containing wt Dph1 did not grow (sensitive to DT, diphthamide formed). The results suggest that all three Cys residues are required for diphthamide biosynthesis. Therefore, the cluster in Dph1 is required for diphthamide biosynthesis in vivo (Fig. 3a).

Based on *Ph*Dph2, the corresponding iron–sulfur cluster-binding residues in yeast Dph2 are Cys106 and Cys362. We also include several more cysteine residues in yeast Dph2. Cys to Ala mutations were made for each of these Cys residues, and the same in vivo assay was employed to evaluate whether these Cys residues were essential for the activity. The result showed that Cys106 is not required for activity, but C107, C128 and C362 are required for activity (Fig. 3b). Notably, C128 is conserved among the eukaryotic Dph1 and Dph2 genes, but not in *Ph*Dph2. The data suggest that the cluster in eukaryotic Dph2 is also required for diphthamide biosynthesis in vivo.

The Fe–S cluster in Dph1 is the catalytic site

We previously reported that yeast Dph3 binds iron and has redox activity [12]. With the *E. coli* flavin protein NorW, the Dph3/NorW/NADH system can reduce Dph1–Dph2 and reconstitute the first step of diphthamide biosynthesis in vitro. We later also found that yeast protein Cbr1 is the endogenous reductase of Dph1–Dph2; the Dph3/Cbr1/NADH system can not only reduce Dph1–Dph2 in diphthamide biosynthesis, but also the radical SAM enzyme Elp3 in tRNA modification [17]. Given that Dph1–Dph2 likely has two Fe–S clusters with Dph1 and Dph2 each has one, the function of each Fe–S cluster became interesting.

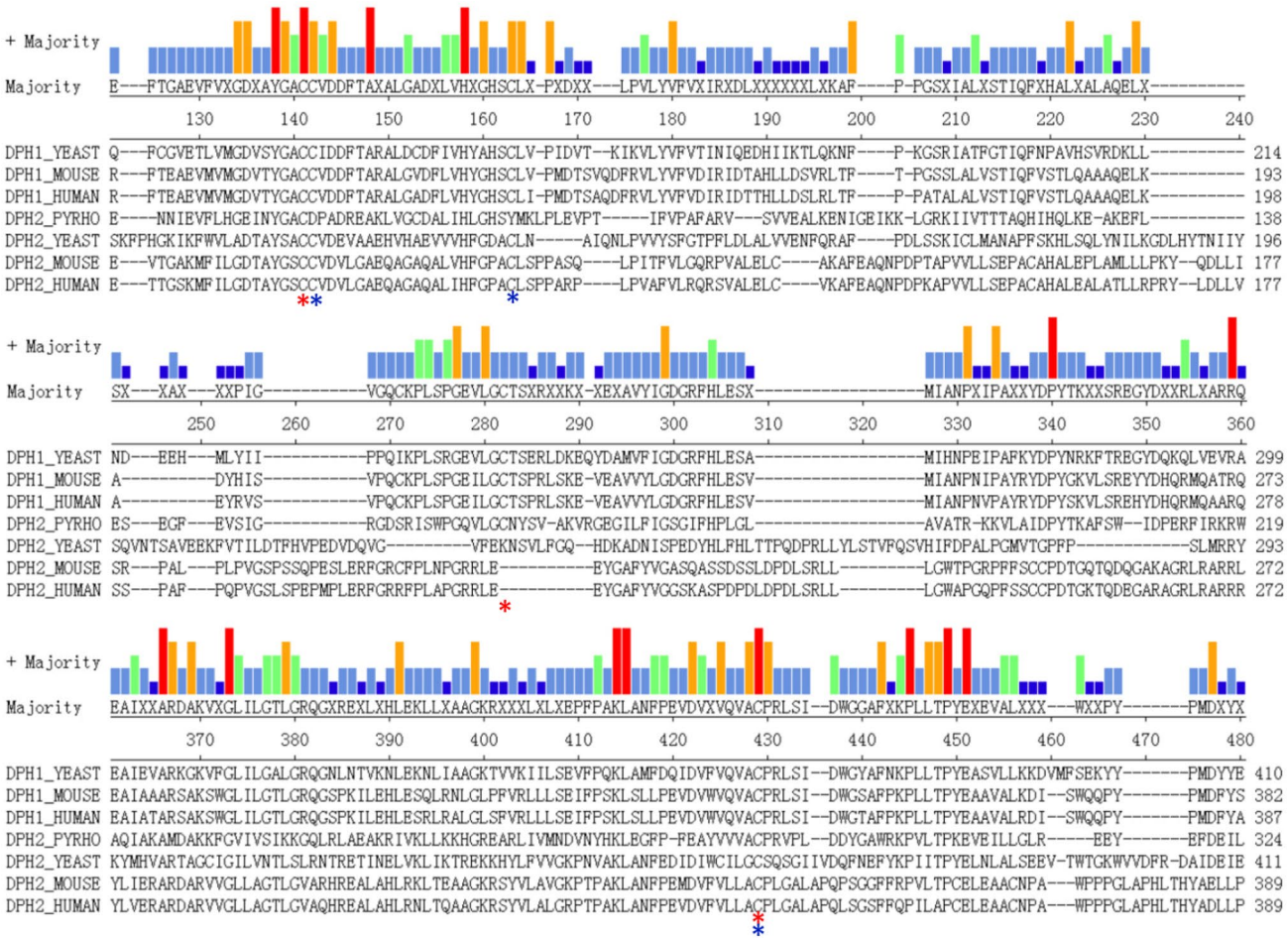

Fig. 2 Sequence alignment of Dph1 and Dph2 proteins from different species. Red "*" indicates the cysteine residues that are required (binding to the [4Fe–4S] cluster) in *Ph*Dph2 and yeast Dph1, while blue "*" indicates cysteine residues important for yeast Dph2. Color code of the colored bars above the aligned sequences: Conservative degree decreases by the sequence of red (absolutely conserved), orange, green, cyan and blue (not conserved)

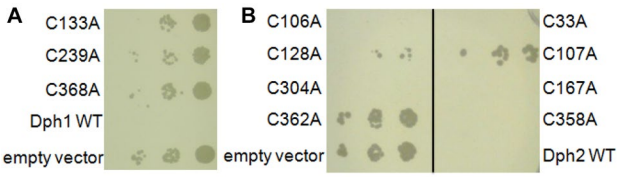

Fig. 3 Yeast Dph1 and Dph2 each has three Cys residues required for activity. **a** Δ*dph1* strain expressing DT was transformed with either an empty vector or vectors encoding wild type (WT) or Cys mutants of Dph1. Each row represents a serial dilution from right to left. Dph1 WT catalyzes diphthamide biosynthesis, leading to cell killing by DT. If a Cys residue is required for activity, then the cells expressing the Cys mutant will not make diphthamide and thus can survive DT. **b** The same assay for Dph2 Cys mutants in Δ*dph2* yeast strain. C128, C362, and C107 are required for diphthamide biosynthesis, while C33, C106, C167, C304, and C358 are not

We hypothesized that one of the Fe–S cluster may be the catalytic site, while the other may facilitate the reduction by Dph3/Cbr1/NADH.

To further investigate the function of each unit, we prepared the Dph1–Dph2 heterodimers with mutations on either Dph1 or Dph2 and investigated their activity in vitro. At first, cysteine to alanine mutants were expressed and purified, but very little protein was recovered. We were concerned that the change of cysteine to alanine may be too disruptive and lead to decreased protein stability. Thus, we prepared corresponding cysteine to serine mutants and tested these mutants with the in vivo DT assay to see if they can still disrupt diphthamide biosynthesis. Mutation of any of the three cysteine residues (C133, C239, and C368) to serine in Dph1 disrupted diphthamide biosynthesis (Fig. 4a), which is consistent with the results of the alanine mutants. However, for Dph2, only mutation of C362 to serine disrupted diphthamide biosynthesis among the three cysteine residues

(C107, C128, and C362) (Fig. 4b). This is different from the alanine mutants shown in Fig. 3.

From our *Ph*Dph2 work, single cysteine mutation of *Ph*Dph2 is not sufficient to disrupt the iron–sulfur cluster; however, double cysteine mutation of *Ph*Dph2 does disrupt the function [15]. To make sure that we completely disrupt the Fe–S cluster, we prepared double cysteine to serine mutants of Dph1 (C239S/C368S) and Dph2 (C107S/C362S). We abbreviated these mutants as Dph1M and Dph2M, respectively. Even with the double Cys-to-Ser mutants, we were able to obtain the recombinant Dph1–Dph2 heterodimer. EPR spectra show that both Dph1M–Dph2 and Dph1–Dph2M heterodimers have [4Fe–4S] cluster (Fig. 5). This result more convincingly demonstrates that yeast Dph1 and Dph2 each can bind a [4Fe–4S] cluster.

With the two mutant Dph1–Dph2 heterodimers containing only one cluster in either Dph1 or Dph2, we then investigated their ability to catalyze the first step of diphthamide biosynthesis in vitro. Our hypothesis was that if the mutated cluster is a catalytic one, it should not have any activity even if dithionite is used as the reductant. In contrast, if the cluster facilitates the reduction of the catalytic cluster, the mutant Dph1–Dph2 heterodimer will be inactive with Dph3/Cbr1/NADH as the reductant, but may still show catalytic activity when dithionite is used as the reductant.

We used carboxyl-^{14}C-SAM for the in vitro reconstitution of the first step of diphthamide biosynthesis. When Dph1M–Dph2 was used, no activity was detected using dithionite or the Dph3/Cbr1/NADH system as the reductant (Fig. 6). When Dph1–Dph2M was used, no product was detected when the Dph3/Cbr1/NADH system was used as the reductant, but the product was formed when using dithionite as a reductant. These results indicated that the cluster in Dph1 unit had catalytic activity, while the cluster in Dph2 is important for the Dph3/Cbr1/NADH system to reduce the catalytic Dph1 cluster.

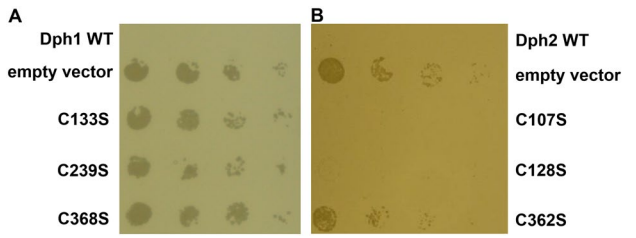

Fig. 4 DT assay for Cys to Ser mutants of Dph1 and Dph2. **a** The Δ*dph1* yeast strains were transformed with either empty vector or vector encoding Cys to Ser mutants of Dph1. Each row represents a serial dilution from left to right. **b** The Δ*dph2* yeast strains were transformed with either empty vector or vector encoding Cys to Ser mutants of Dph2. Each row represents a serial dilution from left to right

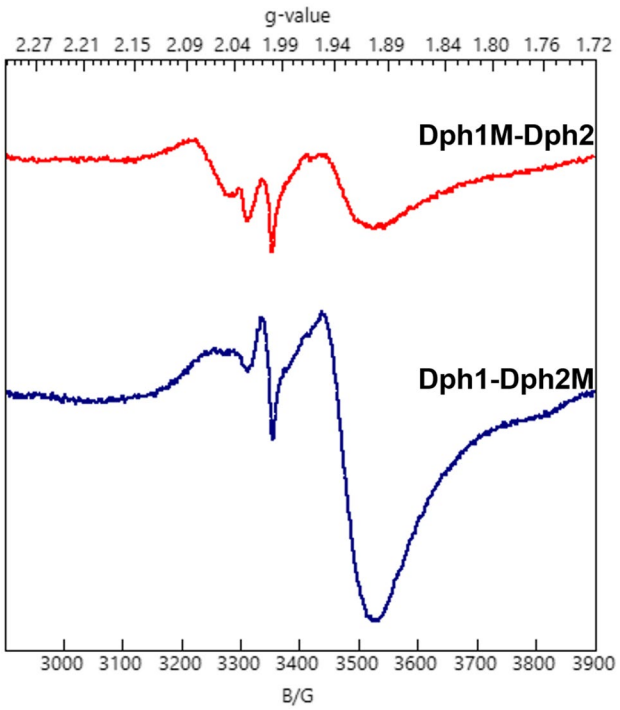

Fig. 5 X-band EPR spectra of Dph1–Dph2 mutants at 12 K. Dph1 (C239S, C368S)–Dph2 is labeled as Dph1M–Dph2. Dph1–Dph2 (C107S, C362S) is labeled as Dph1–Dph2M. EPR spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; modulation amplitude, 8 G; modulation frequency, 100 kHz; microwave power, 0.63 mW. The sharp signal around 3340 G is from a fraction of 2Fe–2S cluster

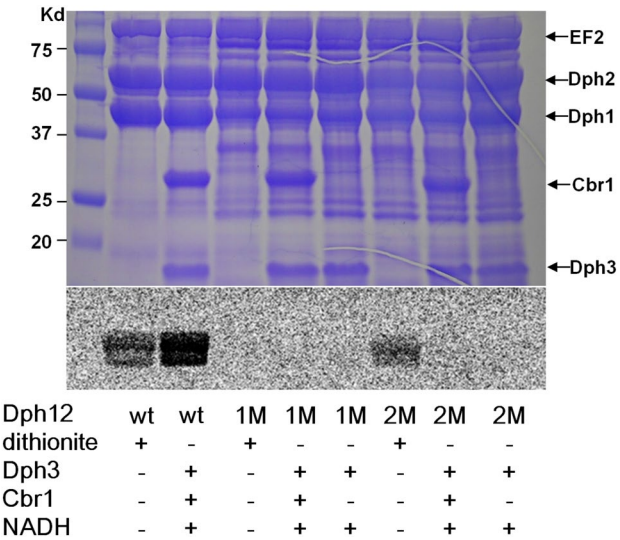

Fig. 6 The ^{14}C-SAM labeling assay of EF2 to detect Dph1–Dph2 activity. The top panel displays the Coomassie blue-stained SDS-PAGE gel for the reaction mixtures. The bottom panel displays the autoradiography detecting ^{14}C-labeled EF2. Mutant Dph1M–Dph2 is labeled as 1 M. Dph1–Dph2M is labeled as 2M

Conclusions

In the present work, we show that yeast Dph1 and Dph2 each has conserved cysteines to bind an Fe–S cluster. The cluster in Dph1 is the catalytic site. With the cysteine mutations that disrupt the cluster in Dph1, the cluster in Dph2 cannot catalyze the reaction in the ^{14}C-SAM labeling assay, no matter whether dithionite or Dph3/Cbr1/NADH is used as the reductant. With the cysteine mutants that disrupt the cluster in Dph2, the cluster in Dph1 can be reduced by dithionite and catalyze the reaction in the ^{14}C-SAM labeling assay. However, the Dph3/Cbr1/NADH system cannot reduce the cluster in Dph1 without the cluster in Dph2. Therefore, our work suggests that in eukaryotic diphthamide biosynthesis, the cluster in Dph2 facilitates the electron transfer from Dph3/Cbr1/NADH to the cluster in Dph1.

Although we cannot completely rule out other possibilities at this point, our EPR data suggest that the Fe–S cluster in Dph2 is likely a [4Fe–4S]. Since this cluster may not bind SAM, this also raises the question whether a fourth ligand (in addition to Cys107, Cys128, and Cys362) is present in Dph2 to coordinate the [4Fe–4S] cluster. A possible candidate for the four ligands is Cys106, since it is right next Cys107 and could be close enough to serve as the fourth ligand. Another possibility is that Dph2 could still bind SAM and thus a fourth ligand is not required. Future biochemical and structural studies will be required to distinguish these possibilities.

Reduction of 4Fe–4S clusters in radical SAM enzyme by biological reductants is a process that is not well understood, especially because the reduction potential is estimated to be around − 500 mV, much lower than that of biological reductants [18]. Our finding that the Dph2 cluster serves to facilitate the reduction of the catalytic cluster may represent an opportunity to study the reduction of 4Fe–4S cluster in radical SAM enzymes.

Acknowledgements This work was supported by grants from the National Institutes of Health National Institute of General Medical Sciences GM088276 to H.L.

References

1. Van Ness BG, Howard JB, Bodley JW (1980) J Biol Chem 255:10710
2. Collier RJ (2001) Toxicon 39:1793
3. Chen C-M, Behringer RR (2005) Curr Opin Genet Dev 15:49
4. Su X, Lin Z, Lin H (2013) Crit Rev Biochem Mol Biol 48:515
5. Schaffrath R, Abdel-Fattah W, Klassen R, Stark MJ (2014) Mol Microbiol 94:1213
6. Tsuda-Sakurai K, Miura M (2019) J Biochem 165:1
7. Dong M, Zhang Y, Lin H (2018) Biochemistry 57:3454
8. Lin Z, Su X, Chen W, Ci B, Zhang S, Lin H (2014) J Am Chem Soc 136:6179
9. Su X, Chen W, Lee W, Jiang H, Zhang S, Lin H (2012) J Am Chem Soc 134:773
10. Su X, Lin Z, Chen W, Jiang H, Zhang S, Lin H (2012) Proc Natl Acad Sci USA 109:19983
11. Uthman S, Bar C, Scheidt V, Liu S, ten Have S, Giorgini F, Stark MJ, Schaffrath R (2013) PLoS Genet 9:e1003334
12. Dong M, Su X, Dzikovski B, Dando EE, Zhu X, Du J, Freed JH, Lin H (2014) J Am Chem Soc 136:1754
13. Zhang Y, Zhu X, Torelli AT, Lee M, Dzikovski B, Koralewski RM, Wang E, Freed J, Krebs C, Ealick SE, Lin H (2010) Nature 465:891
14. Dong M, Kathiresan V, Fenwick MK, Torelli AT, Zhang Y, Caranto JD, Dzikovski B, Sharma A, Lancaster KM, Freed JH, Ealick SE, Hoffman BM, Lin H (2018) Science 359:1247
15. Zhu X, Dzikovski B, Su X, Torelli AT, Zhang Y, Ealick SE, Freed JH, Lin H (2011) Mol BioSyst 7:74
16. Dong M, Horitani M, Dzikovski B, Pandelia ME, Krebs C, Freed JH, Hoffman BM, Lin H (2016) J Am Chem Soc 138:9755
17. Lin Z, Dong M, Zhang Y, Lee EA, Lin H (2016) Nat Chem Biol 12:995
18. Broderick JB, Duffus BR, Duschene KS, Shepard EM (2014) Chem Rev 114:4229