Diphthamide is a post-translationally modified histidine residue present on archael and eukaryotic elongation factor 2 (eEF-2), a GTPase involved in the translocation of mRNA and tRNA on the ribosome during translation elongation. This exceptional modification is targeted by the pathogenic bacterium, Corynebacterium diphtheriae, which causes the infectious disease diphtheria in humans. Diphtheria toxin (DT) produced by this bacterium catalyzes the ADP-ribosylation of the diphthamide residue of eEF-2 using nicotinamide adenine dinucleotide (NAD) as the ADP-ribosyl donor. Irreversible ADP-ribosylation inactivates eEF-2, which in turn stops translation, leading to cell death. Diphthamide is reported to be important for preventing −1 translational frame shift in yeast and mammalian cells. Intriguingly, this modification is not present in EF-G, the bacterial ortholog of eEF-2.

Genetic and biochemical studies in the budding yeast Saccharomyces cerevisiae allowed dissection of the diphthamide biosynthesis pathway. It was initially proposed that the biosynthesis involves three steps (Scheme 1A). Four proteins, Dph1−4, are required for the first step, which involves the transfer of the 3-amino-3-carboxypropyl (ACP) group from S-adenosyl methionine (SAM) to the C2 carbon of the imidazole ring of His699 of yeast eEF-2 (His715 of mammalian eEF-2). Recent evidence suggests that this step uses a unique [4Fe-4S]-containing enzyme and a radical reaction mechanism. The second step involves a single methyltransferase, Dph5, which catalyzes the trimethylation of the amino group to form diphthine (2) through a radical reaction mechanism. The third step is between the Dph5- and Dph6-catalyzed reactions. We demonstrate that the Dph5-catalyzed reaction generates methyalted diphthine, a previously overlooked intermediate, and Dph7 is a methylesterase that hydrolyzes methylated diphthine to produce diphthine and allows the Dph6-catalyzed amidation reaction to occur. Thus, our study characterizes the molecular function of Dph7 for the first time and provides a revised diphthamide biosynthesis pathway.

The exact molecular role of Dph7 is unclear. Here we demonstrate that Dph7 is an enzyme catalyzing a previously unknown step in the diphthamide biosynthesis pathway. This step is between the Dph5- and Dph6-catalyzed reactions. We demonstrate that the Dph5-catalyzed reaction generates methylated diphthine, a previously overlooked intermediate, and Dph7 is a methylesterase that hydrolyzes methylated diphthine to produce diphthine and allows the Dph6-catalyzed amidation reaction to occur. Thus, our study characterizes the molecular function of Dph7 for the first time and provides a revised diphthamide biosynthesis pathway.

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**ABSTRACT:** Present on archael and eukaryotic translation elongation factor 2, diphthamide represents one of the most intriguing post-translational modifications on proteins. The biosynthesis of diphthamide was proposed to occur in three steps requiring seven proteins, Dph1−7, in eukaryotes. The functional assignments of Dph1−5 in the first and second step have been well established. Recent studies suggest that Dph6 (yeast YLR143W or human ATPBD4) and Dph7 (yeast YLR143W or human WDR85) are involved in the last amidation step, with Dph6 being the actual diphthamide synthetase catalyzing the ATP-dependent amidation reaction. However, the exact molecular role of Dph7 is unclear. Here we demonstrate that Dph7 is an enzyme catalyzing a previously unknown step in the diphthamide biosynthesis pathway. This step is between the Dph5- and Dph6-catalyzed reactions. We demonstrate that the Dph5-catalyzed reaction generates methylated diphthine, a previously overlooked intermediate, and Dph7 is a methylesterase that hydrolyzes methylated diphthine to produce diphthine and allows the Dph6-catalyzed amidation reaction to occur. Thus, our study characterizes the molecular function of Dph7 for the first time and provides a revised diphthamide biosynthesis pathway.
cell line with Dph7 gene deletion.\textsuperscript{20} This methylation was thought to occur on one of the nitrogen atoms of the imidazole ring of the histidine residue (3, Figure 1).\textsuperscript{20} Here we demonstrate that this modification is actually methylated diphthine with the methyl group on the carbonyl of diphthine (4a, Figure 1) and Dph7 is a methylesterase responsible for the hydrolysis of the methylated diphthine (4a) to generate diphthine (2), which can then be used by Dph6 in the last amidation step (Scheme 1B). Methylated diphthine (4a) is produced by the enzymatic function of Dph6. The present work thus uncoils the molecular function of Dph7 and provides a revised diphthamide biosynthesis pathway (Scheme 1B).

To determine if Dph7 had an enzymatic role or merely mediated the interaction of Dph6 with eEF-2, we purified eEF-2 proteins from a yeast strain with Dph6 deletion (Δdph6) or a yeast strain with Dph7 deletion (Δdph7) for in vitro reconstitution of the amidation reaction. The purified eEF-2 proteins were incubated with Dph6, ATP, and ammonium chloride for amidation. Diphthamide formation was detected with fluorescently labeled rhodamine-NAD (Rh-NAD) and a fluorescently labeled cyanine 3 (Cy3). The results of this investigation suggested a possible enzymatic role for Dph7 as a demethylase. We hypothesized that Dph7 functions to remove the extra methyl group on methylated diphthine (4a or 4b) in Δdph6 eEF-2 and Δdph7 eEF-2 via liquid chromatography-mass spectrometry (LC-MS) studies. Consistent with previous studies, we found diphthine (2) containing tryptic peptide (686-VNLDVTLDHADHR-700) from both Δdph7 and Δdph6 eEF-2 samples (Figure S1A). Likewise, we observed a small amount of unmodified peptides, but no diphthamide (1) was detected in either eEF-2 sample.\textsuperscript{16,18,19} We also found the presence of methylated diphthine (4a or 4b) in Δdph7 eEF-2 (Figure S1B). Most strikingly, this methylated diphthine (4a or 4b) was not detected in Δdph6 eEF-2. This unexpected form of modification had an m/z larger than those of all the previously known intermediates of diphthamide biosynthesis or diphthamide (1). The results of this investigation suggested a possible enzymatic role for Dph7 as a demethylase. We hypothesized that Dph7 functions to remove the extra methyl group on methylated diphthine (4a or 4b) to form diphthine (2).

To further demonstrate that Dph7 catalyzes an additional step, we incubated flag-tagged Δdph7 eEF-2 with Dph7 and repurified the Δdph7 eEF-2 to remove Dph7. We found that the repurified Δdph7 eEF-2 was a substrate for the amidation by Dph6 alone (Figure 2B, lane 2). In contrast, flag-tagged Δdph7 eEF-2 incubated without Dph7 did not form diphthamide (Figure 2B, lane 1). Taken together, these findings indicate that there is an additional step before the last amidation step in the diphthamide biosynthesis and Dph7 is the enzyme catalyzing this step.

The conclusion of Dph7 having enzymatic function is seemingly contradictory to previous reports showing that Δdph6 eEF-2 and Δdph7 eEF-2 both contain diphthine (2). Based on these observations, there is no room for any apparent chemical transformation for Dph7’s enzymatic activity. Interestingly, a species with a mass of 15 Da larger than that of diphthamide (1) was reported in a lymphoma cell line with Dph7 gene deletion.\textsuperscript{19} The expected mass difference of 3 and 1 is 14 Da. Therefore, we speculated that the observed species was methylated diphthine instead (4a or 4b, Figure 1). In light of this report, we investigated the presence of methylated diphthine (4a or 4b) in Δdph7 eEF-2 and Δdph6 eEF-2 via liquid chromatography-mass spectrometry (LC-MS) studies. Consistent with previous studies, we found diphthine (2) containing tryptic peptide (686-VNLDVTLDHADHR-700) from both Δdph7 and Δdph6 eEF-2 samples (Figure S1A). Likewise, we observed a small amount of unmodified peptides, but no diphthamide (1) was detected in either eEF-2 sample.\textsuperscript{16,18,19} We also found that methylated diphthine (4a or 4b) was not detected in Δdph6 eEF-2. This unexpected form of modification had an m/z larger than those of all the previously known intermediates of diphthamide biosynthesis or diphthamide (1). The results of this investigation suggested a possible enzymatic role for Dph7 as a demethylase. We hypothesized that Dph7 functions to remove the extra methyl group on methylated diphthine (4a or 4b) to form diphthine (2). Furthermore, the fact that 2 was also observed in MS studies of Δdph7 eEF-2 suggested that this methyl group was relatively labile during the sample preparation for MS. Therefore, we proposed that methylated diphthine is a methyl ester which is prone to hydrolysis (4a, Scheme 1B).

To test the hypothesis that methylated diphthine (4a or 4b) is a methyl ester (4a), we examined the nonenzymatic hydrolysis of the methyl ester under mild basic conditions. Purified Δdph7 eEF-2 in Tris-HCl pH 9.0 buffer was incubated at 30 °C for various time intervals. The conversion of 4a to 2 was monitored by the amidation reaction, as Dph6 selectively amidates 2, but not 4a, to form diphthamide (1). The formation of 1 is then detected by a fluorescence label using Rh-NAD and DT. As shown in Figure 3A, an increased incubation time for Δdph7 eEF-2 leads to an increased fluorescence label, indicating the time-dependent conversion of 4a to 2. In contrast, extending the incubation time for Δdph6 eEF-2, which contains 2, has no effect...
nonenzymatic hydrolysis of methylated diphthine to diphthine (Figure 4, Figure S2G). Consistent with previous MS analysis, a significant amount of 2 is present in the Δdph5 Δdph5 eEF-2 sample without Dph5, possibly due to nonenzymatic hydrolysis of the methyl ester (4a) during incubation in pH 8.0 buffer and during the sample preparation process for MS analysis.

Our finding that Dph7 is a methylesterase converting methylated diphthine (4a) to diphthine (2) suggests that the diphthamide biosynthesis pathway needs to be revised. In the current literature, 2 is proposed as the product of the second step catalyzed by the methyltransferase, Dph5. It was proposed after the fact that acid hydrolysis of eEF-2 with in vitro reconstitution of the second step yields 2. However, under such conditions, it was likely that 4a, a methyl ester, was hydrolyzed to 2 and was not detected. To investigate if 4a is produced by Dph5 in the second step, we reconstituted the reaction in vitro using purified eEF-2 from the Δdph5 yeast strain, SAM, and purified Dph5 protein. Δdph5 eEF-2 incubated with SAM but without Dph5 was used as a control. The Δdph5 strain is deficient in the second step of diphthamide biosynthesis, and therefore the eEF2 contains 3-amino-3-carboxypropyl (ACP) modified Hist509, the product of the first step. Both experimental and control eEF-2 samples were trypsin-digested and subjected to LC-MS analysis. In agreement with previous MS reports, we found ACP-modified peptide and unmodified peptide, but not other intermediates in the Δdph5 eEF-2 sample without Dph5. For the Δdph5 eEF-2 sample treated with Dph5 and SAM, the level of ACP-modified peptide was considerably lower than that of the control, indicating that the ACP-modified eEF-2 was consumed (Figure 5, Figure S3C and S3D). In addition, we found three other types of modifications on Hist509 of the tryptic peptide (686-VNILDVTL-HADAIHR-700): monomethylated ACP (Figure S3F), diphthine (Figure S3H), and methylated diphthine (Figure S5). These three modified forms were not present in the control sample without Dph5 (Figure S3E, S3G, and S3I). The monomethylated ACP-modified eEF-2 was likely an intermediate for the formation of methylated diphthine. Diphthine (2) was again observed, probably due to hydrolysis of 4a. Thus, the MS study demonstrated that 4a is the product of the second step in diphthamide biosynthesis and Dph5 is responsible for the extra methylation.

In summary, our results presented here demonstrate that there is a previously unidentified step in the diphthamide biosynthesis pathway, and we propose a revised scheme of the diphthamide biosynthesis pathway (Scheme 1B). Yeast Dph5 catalyzes the methylation of the amino and the carboxylate groups of ACP, generating methylated diphthine (4a). The molecular function of Dph7 is to convert methylated diphthine (4a) to diphthine (2) so that Dph6 can convert it to diphthamide (1). Although a considerable amount of 2 was observed in both the MS studies of Δdph7 Δdph7 eEF-2 and Δdph5 Δdph5 eEF-2 treated with Dph5, we believe that 4a is the predominant intermediate formed in vivo. This is because, in the yeast Dph7 deletion strain, if both 2 and 4a are formed by Dph5, any 2 formed will be converted to 1 due to the presence of Dph6. Since 1 was not observed in the MS analysis of Δdph5 Δdph5 eEF-2 (as we and others previously reported), we believe that only 4a is formed by yeast Dph5 in vivo. The observation of 2 in Δdph7 Δdph7 eEF-2 during MS analysis is likely due to the hydrolysis of 4a in the sample preparation process.
Previously, we also reported that archael Dph5 catalyzes N-trimethylation of the ACP group, which leads to the elimination of the trimethylamino group. This elimination reaction was not observed in the yeast Dph5-catalyzed reaction. In contrast, the yeast Dph5 catalyzes tetramethylation of the ACP group. Thus, it seems that archael and eukaryotic diphthamide biosyntheses differ in the methylation step. Consistent with this, archael lacks the demethylation enzyme, Dph7. Methyltransferases are known to be able to transfer methyl groups from SAM to diverse acceptor substrates, and methyl transfer to amino and carboxylate groups are known. Certain N-methyltransferases and O-methyltransferases are known to be promiscuous. However, the remarkable promiscuous methylation activity (both N- and O-methylation) of yeast Dph5 has not been observed in other methyltransferases before.

The functional implication of this additional methylation—demethylation step is still unclear at this point. Since archael Dph5 does not catalyze the extra methylation, it is hard to believe that this extra methylation is merely a side reaction due to eukaryotic Dph5’s lack of specificity. It is more likely that the extra methylation—demethylation step introduced by the promiscuous methylation activity of eukaryotic Dph5 and the methylesterase Dph7 emerged in the evolution process for a certain purpose. It is possible that this extra methylation introduces a blockage to the pathway, creating a regulatory point for diphthamide biosynthesis. The possibility of a regulation on diphthamide biosynthesis via Dph7 awaits further studies.

To the best of our knowledge, Dph7 is the first WD40 protein to have an enzymatic function. Multiple sequence alignments with Dph7 orthologs reveal conserved serine, aspartic acid, and histidine residues that can potentially form a catalytic Ser-His-Asp triad, commonly found in α/β hydrolases (Figure S4). However, due to the presence of WD40 repeats, Dph7 is predicted to adopt a circularized β-propeller structure, lacking the usual α/β hydrolase fold. Pectin methylsterase, which also lacks the α/β hydrolase fold, was demonstrated to adopt a novel esterase active site with two catalytic aspartic residues. Thus, it is possible that the catalytic residues of Dph7 differ from the conventional catalytic triad of α/β hydrolases. It will be interesting to investigate the catalytic mechanism of Dph7 in future studies.

**ASSOCIATED CONTENT**

*Supporting Information*

Experimental materials and methods, supporting figures, and a list of strains used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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