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THE BIOSYNTHESIS AND BIOLOGICAL FUNCTION OF DIPHTHAMIDE

Xiaoyang Su¹, Zhewang Lin¹, and Hening Lin^{1,*}

¹Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14850, USA

Abstract

Eukaryotic and archaeal elongation factor 2 contains a unique post-translationally modified histidine residue, named diphthamide. Genetic and biochemical studies have revealed that diphthamide biosynthesis involves a multi-step pathway that is evolutionally conserved among lower and higher eukaryotes. During certain bacterial infections, diphthamide is specifically recognized by bacterial toxins, including diphtheria toxin, *Pseudomonas* exotoxin A, and cholera toxin. Although the pathological relevance is well studied, the physiological function of diphthamide is still poorly understood. Recently, many new interesting developments in understanding the biosynthesis have been reported. Here, we review the current understanding of the biosynthesis and biological function of diphthamide.

Keywords

diphthamide; post-translational modifications; radical SAM enzyme; diphtheria toxin; diphthine

Introduction

Diphthamide is a post-translationally modified histidine residue found in eukaryotic and archaeal translation elongation factor 2 (EF2). It is named after the fact that it is the target of diphtheria toxin (DT) produced by *Corynebacterium diphtheriae*. DT and the *Pseudomonas aeruginosa* exotoxin A (ETA) catalyze the ADP-ribosylation reaction on the diphthamide residue of EF2 using nicotinamide adenine dinucleotide (NAD) as the ADP-ribosyl donor (Collier, 2001). EF2 catalyzes the translocation of mRNA and peptidyl-tRNA from ribosome A site to P site. The ADP-ribosylation of EF2 inactivates it, stopping protein synthesis and leading to cell death (Honjo et al., 1968, Jorgensen et al., 2006).

Structure determination of diphthamide and the identification of DPH genes

The study of diphthamide starts with the elucidation of the DT modification site. It is first discovered that the target residue of DT is not an ordinary amino acid residue (Robinson et al., 1974). It is further revealed that diphthamide exists only on EF2 and there is one

Corresponding author: Prof. Hening Lin, Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, NY 14850, USA, Phone: 607-255-4650, hl379@cornell.edu.

Declaration of Interest

The authors declare that they have no conflict of interest.

diphthamide residue per EF2 protein (Van Ness et al., 1978). The modification sites are determined in both rat and yeast. The surrounding residues are found to be highly conserved (Van Ness et al., 1978). The structure of ADP-ribosyl diphthamide is determined using NMR and later confirmed by fast atom bombardment mass spectrometry (Van Ness et al., 1980b, Van Ness et al., 1980a). The structure of diphthamide is proposed to be 2-[3-carboxyamido-3-(trimethylammonio)propyl]histidine (Figure 1). This structure has been confirmed by X-ray crystallography (Jorgensen et al., 2004, Jorgensen et al., 2006). However, there is a discrepancy on the stereochemistry of diphthamide. The reported crystal structure of the EF2 suggests an R configuration on the third carbon (marked by an asterisk in Figure 1) of diphthamide side chain (Jorgensen et al., 2004, Jorgensen et al., 2006, Jorgensen et al., 2008a). This assignment of carbon chirality is surprising because the currently proposed biosynthetic pathway indicates that this carbon is originally the α -carbon of a methionine molecule and the S configuration of methionine should be retained in diphthamide. A higher resolution structure is needed to further confirm this unexpected chirality assignment. If this turns out to be true, an update in the diphthamide biosynthetic pathway will be necessary.

The structural determination has facilitated the elucidation of the diphthamide biosynthetic pathway. Using biosynthetic precursor labeling techniques, it becomes clear that the backbone and the three methyl groups of diphthamide come from S-adenosylmethionine (SAM) (Dunlop and Bodley, 1983). The study of DT resistant mutants of CHO-K1 Chinese hamster ovary cells reveals that there are a minimum of three steps (Moehring et al., 1984). A genetic screening method to detect diphthamide deficient strains is developed based on the DT sensitivity of the yeast spheroplasts (Chen et al., 1985). Five complementation groups are found and they are designated *dph1*, *dph2*, *dph3*, *dph4* and *dph5*. It becomes clear later that Dph1-4 are responsible for the first step (Chen and Bodley, 1988) (Figure 2), which involves the transfer of the 3-amino-3-carboxypropyl (ACP) group. Dph5 is a methyltransferase that produces the diphthine intermediate (Chen and Bodley, 1988). Diphthine can be ADP-ribosylated, but the reaction rate is only 3% of that of the diphthamide ADP-ribosylation reaction (Chen and Bodley, 1988). It is also suggested that there is a diphthine amidation step following Dph5-catalyzed trimethylation (Figure 2). But the amidation enzyme or diphthamide synthetase remains unknown for almost three decades until their identification very recently. It has been speculated that the genetic screening used could not identify diphthamide synthetase because diphthine can be ADP-ribosylated by DT.

A breakthrough comes when human gene WDR85, which is the ortholog of yeast gene YBR246W, is identified as a diphthamide biosynthetic gene (hence later named Dph7) using haploid genetic screening (Carette et al., 2009). Later study show that Dph7 is required for the conversion from diphthine to diphthamide (Su et al., 2012a). The reason that Carette et al. can identify Dph7 is likely because they use ETA instead of DT. ETA has a better ability to differentiate diphthine and diphthamide due to the presence of an aspartate residue (Asp461) instead of an asparagine residue (Asn46) in DT (Su et al., 2012b) (Jorgensen et al., 2008a). The carboxylate of Asp461 interacts favorably with the diphthamide amide group, but will repel the carboxylate of diphthine due to electrostatics. In contrast, in DT, the neutral Asn46 residue will not repel the carboxylate of diphthine. Cholix toxin is another

bacterial toxin that ADP-ribosylates EF2 (Jorgensen et al., 2008b). It also possesses an acidic residue, Glu484, to discriminate between diphthamide and diphthine. It is not clear why such discrimination is not present in DT.

Following the identification of Dph7, yeast gene YLR143W is identified as the diphthamide synthetase, which catalyzes the amidation reaction (Su et al., 2012b). Two more independent research groups come to the same conclusion via different routes and the common name Dph6 is suggested for YLR143W (de Crecy-Lagard et al., 2012, Uthman et al., 2013). All of these studies take the advantage of the large scale functional genomic data in yeast that is publicly available, such as the yeast growth fitness database (Su et al., 2012b). These studies provide an interesting example on how large scale functional genomic data can be used to identify missing components in a defined biochemical pathway.

Evolutionarily, the diphthamide biosynthetic pathway develops in two phases. *P. horikoshii* and most other sequenced archaeal species have the orthologs of Dph2, Dph5 and Dph6 (de Crecy-Lagard et al., 2012). However, in one species of the deep-branching phylum *Korarchaeota*, these three DPH genes are absent. In fact, this organism lacks only five genes that are represented in all sequenced archaeal genomes, namely, orthologs of Dph2, Dph5 and Dph6, a predicted Zn-ribbon RNA-binding protein, and a small-conductance mechanosensitive channel (Elkins et al., 2008). This means that the most essential part of this pathway emerges all at once. Other Dph genes are conserved in eukaryotes but are absent in archaea.

Molecular function of the DPH genes

The first step of diphthamide biosynthesis is the transfer of the ACP group from SAM to the C2 position of the imidazole ring of the histidine residue being modified in EF2. This reaction has been reconstituted *in vitro* and studied in great detail using the enzyme from an archaeal species, *Pyrococcus horikoshii* (Zhang et al., 2010). Unlike yeast, *P. horikoshii* has only one protein, Dph2, identified for the first step. The *P. horikoshii* Dph2 (PhDph2) is homologous to eukaryotic Dph1 and Dph2. Crystal structure of PhDph2 has been solved. PhDph2 forms a homodimer and each monomer consists of three domains with similar folding patterns (Figure 3). The basic domain folding pattern is a four-stranded parallel β -sheet with three flanking α -helices (Zhang et al., 2010). Although PhDph2 lacks the CX3CX2C motif which is present in most radical SAM enzymes (Frey et al., 2008), it contains three cysteine residues (Cys59, Cys163 and Cys287) that are spatially close (Figure 3).

The structural observation has led to the discovery that PhDph2 coordinates a [4Fe-4S] cluster and is an unusual radical SAM enzyme. Unlike most other radical SAM enzymes, PhDph2 does not generate a 5'-deoxyadenosyl radical (Figure 4). Instead, PhDph2 cleaves the C_γ,Met-S bond of SAM to form 5'-deoxy-5'-methylthioadenosine (MTA) and an ACP radical (Zhu et al., 2011, Lin, 2011). The ACP radical then likely adds to the imidazole ring of the histidine residue to give the product (Figure 4). PhDph2 is the first enzyme known to generate an ACP radical from SAM and this may help to understand how radical SAM enzymes work in general.

In eukaryotes the first step is considerably more complicated than in *P. horikoshii*. There are four genes known to be required for this step, DPH1-4. Dph1 and Dph2 are homologous to each other and presumably form a heterodimer that is functionally similar to the PhDph2 homodimer. It has been shown that in the PhDph2 homodimer, only one active monomer is required for the activity *in vitro* (Zhu et al., 2011). PhDph2 has three cysteine residues coordinating the [4Fe-4S] cluster. These three cysteine residues are well conserved in eukaryotic Dph1, while the eukaryotic Dph2 has only the first and the third cysteine residues in place. The functional implication of this asymmetry in Dph1-Dph2 awaits further study.

Dph3 from *Saccharomyces cerevisiae* is only 82 amino acids long. However, there are two domains, an N-terminal cystathionine β -synthase (CBS) domain and a C-terminal Zn ribbon domain (Proudfoot et al., 2008). The solution structure of Dph3 has been reported. It comprises a 3_{10} helix, two turns, two α helices and two β sheets (Sun et al., 2005). The C-terminal Zn ribbon domain has 4 conserved cysteine residues arranged in Cys-X-Cys-X₁₉-Cys-X₂-Cys motif. The Zn ribbon domain is found to bind both zinc and iron. Dph3 can be reduced by *E. coli* rubredoxin reductase NorW, suggesting it may serve as a rubredoxin-like electron carrier (Proudfoot et al., 2008). Dph3 forms a complex with Dph1 and Dph2 (Bar et al., 2008, Abdel-Fattah et al., 2013) and possibly mediates electron transfer to Dph1-Dph2.

Dph3 likely has functions other than diphthamide biosynthesis. *dph3* grows slower compared to other DPH gene deletion strains, implying that it has multiple functions (Bar et al., 2008). So far, people have shown several Dph3 binding partners, including the elongator complex (Fichtner and Schaffrath, 2002, Greenwood et al., 2009), DelGEF (deafness locus associated putative guanine nucleotide exchange factor) (Sjolinder et al., 2004) and yeast homologues of endophilin and amphiphysin (Krogan et al., 2006). The physical interactions of Dph3 imply a variety of physiological functions, spanning from transcription regulation to endocytosis and secretion. The study of biochemical role of Dph3 in diphthamide biosynthesis is likely to shed light on its other cellular functions and vice versa.

Yeast Dph4 has an N-terminal J domain which is commonly found in co-chaperones of 70 kilodalton heat shock proteins (Hsp70). J domain is responsible for the stimulation of the ATPase activity of their partner Hsp70s which is crucial for the interaction of these chaperone machineries with their client proteins. The J domain is required for the activity of Dph4 in diphthamide biosynthesis. However, a chimeric version of Dph4 with the J domain from Ydj1 is also active (Sahi and Craig, 2007). The C-terminal of yeast Dph4 contains a CSL zinc finger (Sahi and Craig, 2007). The cysteine residue in the CSL sequence is also important for the activity of Dph4. The CSL zinc finger in Dph4 is shown to bind Fe more tightly than Zn. Iron binding induces the oligomerization of Dph4. The iron containing Dph4 is redox-active (Thakur et al., 2012). Based on the domain organization, Dph4 may have either electron transfer activity or co-chaperone activity or both. However, unlike Dph3, Dph4 does not co-localize with Dph1 and Dph2 (Webb et al., 2008). This fact suggests that Dph4 is more likely to be a co-chaperone involved in the iron-sulfur cluster assembly on Dph1 and Dph2.

Dph5 is a methyltransferase that catalyzes the trimethylation of the amino group (Mattheakis et al., 1992). The product of this step is named diphthine. Like most other

methyltransferases, Dph5 uses SAM as the methyl group donor. Eukaryotic EF2 (eEF2) from *dph5* has a very weak activity as the substrate for ADP-ribosylation, about 10⁶ times weaker than diphthamide-containing eEF2 from the wild-type strain (Mattheakis et al., 1992). The diphthine-containing eEF2, obtained by *in vitro* methylation using purified Dph5, is a much better substrate for ADP-ribosylation and is only about 30 times weaker than diphthamide-containing eEF2 (Chen and Bodley, 1988). Dph5 interacts with eEF2 and this interaction is enhanced when Dph1 or Dph7 is deleted (Uthman et al., 2013). In addition, overexpression of Dph5 is growth inhibitory to Dph1, Dph2, Dph3, Dph4, or Dph7 null strain (Uthman et al., 2013).

P. horikoshii Dph5 (PhDph5)-catalyzed diphthine formation has been reconstituted *in vitro* using purified proteins (Zhu et al., 2010). PhDph5 is able to catalyze the trimethylation reaction in a highly processive manner. Interestingly, it is found that diphthine on PhDph5 is not stable. It tends to lose the trimethylamino group in a reaction that is similar to Hofmann elimination (Zhu et al., 2010). This reaction does not happen readily on yeast diphthine (Su et al., 2012a). The exact implication of this reaction is unknown. However, it is possible that archaeal diphthamide has a different structure and this may explain the weak activity of archaeal EF2 as a substrate in ADP-ribosylation reactions (Pappenheimer et al., 1983).

Recent work suggests additional methylation could occur to diphthamide. In a lymphoma cell line, when Dph7 (WDR85) is deleted, the cells are resistant to exotoxin A treatment. Interestingly, an additional methylation is found on the EF2 isolated from this cell line (Wei et al., 2013). The methylation is thought to occur on one of the N of the imidazole ring of the histidine residue. It is not clear how the additional methylation happens and whether it is important for the process of normal diphthamide biosynthesis.

Dph6 belongs to the adenine nucleotide alpha hydrolases superfamily. *In vitro* assay suggests that Dph6 consumes ATP and generates AMP (Su et al., 2012b). The direct nitrogen donor in the diphthine amidation reaction is ammonia (Su et al., 2012b). Glutamine cannot serve as the nitrogen donor in this reaction *in vitro*. Yeast Dph6 has two distinctive functional domains. At the N-terminal is an alpha₁ANH₁ domain and at the C-terminal is an Yjg-YER057c-UK114 domain. Both domains are reported to be important for the function of yeast Dph6 (Uthman et al., 2013). Human gene ATP-binding-domain containing protein 4 (ATPBD4) is the ortholog of yeast Dph6. Human ATPBD4 rescues diphthamide biosynthesis in yeast *dph6* strain (Su et al., 2012b). The Yjg-YER057c-UK114 domain is absent in human ATPBD4.

Dph7 is required for the conversion from diphthine to diphthamide. *dph7* yeast strain accumulates diphthine (Su et al., 2012a). Dph7 is a WD40 repeats containing protein and may mediate protein-protein interactions (Su et al., 2012a). However, the precise molecular function of Dph7 in diphthamide biosynthesis awaits further studies. As discussed above, methylated diphthamide is also found in *dph7* (Wei et al., 2013). Dph5 was found to bind eEF2 more tightly in *dph7* (Carette et al., 2009, Uthman et al., 2013). Therefore, it has been proposed that Dph7 is responsible for either displacing Dph5 from EF2 or preventing the additional methylation. Dph7 has also been shown to participate in the retromer-mediated endosomal recycling pathway and named Ere1. Ere1 (Dph7) and Ere2 are

cytoplasmic proteins that are recruited to endosome and assembled into protein complexes on endosome (Shi et al., 2011). It is unclear whether Dph7 plays independent roles in these two seemingly unrelated pathways or not. Understanding the exact molecular function of Dph7 will help addressing this question. A possible connection between diphthamide biosynthesis and endosomal recycling by Dph7 could potentially shed lights on the poorly understood function of diphthamide modification, which will be discussed below.

The biological function of diphthamide

The biological function of diphthamide is still not well understood. Diphthamide modification makes cells susceptible to bacterial toxins, yet it is highly conserved among archaea and eukaryotes. The formation of diphthamide involves multiple proteins and enzymatic steps. It is hard to believe that such a system exists only to be exploited by bacterial toxins. The evolutionary conservation and complexity of diphthamide modification suggest a fundamental role of diphthamide.

Diphthamide has been found to be important in maintaining translational fidelity. The lack of diphthamide results in elevated -1 frameshift in protein synthesis (Ortiz et al., 2006). Diphthamide resides in the tip of domain IV of eEF2 which is in close proximity with the tRNA in the P-site (Spahn et al., 2004). The positive charge incurred by diphthamide modification helps eEF2 to maintain conformational integrity (Liu et al., 2012).

Despite the role of diphthamide in maintaining translation fidelity, in yeast none of the DPH genes is essential. In contrast, many DPH genes are essential in mammals. DPH1 gene has been found to be frequently deleted in ovarian and breast cancer, hence the alias OVCA1 (ovarian cancer gene 1) (Phillips et al., 1996b, Phillips et al., 1996a, Chen and Behringer, 2001). Overexpression of OVCA1 suppresses the colony formation of ovarian cancer cells, presumably by cell cycle arrest at G1 phase (Bruening et al., 1999). It was further demonstrated that OVCA1 overexpression in ovarian cancer cells A2780 leads to down-regulation of cyclin D1, and up-regulation of cyclin-dependent kinase inhibitor p16 (Kong et al., 2011). OVCA1 homozygous mutant mice die at birth due to developmental delay and multiple organ defects while OVCA1 heterozygous mice develop cancer spontaneously (Chen and Behringer, 2004). Similar to DPH1, DPH3 homozygous deletion leads to embryonic death (Liu et al., 2006) and DPH4 homozygous mutants were retarded in growth and development (Webb et al., 2008). These developmental defects has been attributed to the deficiency of diphthamide (Liu et al., 2012). However, it is not clear whether these phenotypes are due to decreased translation fidelity or due to the loss of other unknown functions of diphthamide.

It has long been speculated that diphthamide is the target of endogenous ADP-ribosyltransferases. Bacterial toxin induced ADP-ribosylation shuts down ribosomal protein synthesis and kills host cells. It has been reported that diphthamide can also be ADP-ribosylated by endogenous ADP-ribosyltransferase and this may serve as a regulatory mechanism for cellular protein synthesis (Lee and Iglewski, 1984). The endogenous ADP-ribosyltransferase activity is found in both polyoma virus-transformed baby hamster kidney (pyBHK) cells (Lee and Iglewski, 1984) and beef liver (Iglewski et al., 1984). However,

later studies show that the eEF2 ADP-ribosylation activity from pyBHK cells is independent of diphthamide modification (Fendrick et al., 1992, Muhammet et al., 2006). It has also been reported that interleukin-1 β may act as an activator of the endogenous ADP-ribosylation (Jager et al., 2011). To our best knowledge, there has not been any endogenous diphthamide ADP-ribosyltransferase cloned and characterized.

Summary

Diphthamide represents one of the most intriguing post-translational modifications since its discovery over three decades ago. Remarkably, formation of this conserved modification in archaea and eukaryotes involves multiple enzymatic steps. In eukaryotes, seven genes, Dph1-Dph7, are required for the biosynthesis. Despite the long history of diphthamide research, we are amazed by the recent new findings that remind us about the complexity of this unusual protein post-translational modification. Only in the last several years has the diphthamide synthetase (Dph6) been identified (Su et al., 2012b) (Uthman et al., 2013) and the unusual radical enzyme chemistry been investigated (Zhang et al., 2010). Many questions remain unaddressed in diphthamide biosynthesis. The detailed reaction mechanism of the first step and how PhDph2 or Dph1-Dph2 control the formation of an ACP radical awaits further studies. The molecular function of Dph3, Dph4, and Dph7 are still unknown. The mechanism and function of the additional methylation step recently reported need to be further investigated (Wei et al., 2013). In terms of biological function, it is not clear whether diphthamide has functions other than ensuring translation fidelity. Whether the biosynthesis of diphthamide is regulated and whether it allows translation to be regulated are still open questions. Interestingly, both Dph3 and Dph7 are involved in other biological pathways. Studying the possible connections between diphthamide biosynthesis and these other pathways may provide new insights into the physiological function of diphthamide.

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Figure 1.

The structure of diphthamide and the ADP-ribosylation reaction. The asterisk marks the third carbon of diphthamide side chain whose configuration is in question.

Figure 2.

The proposed biosynthetic pathway of diphthamide. A color version of the figure is available online.

Figure 3.
Structure of PhDph2 homodimer (PDB 3LZD) showing the [4Fe-4S] cluster coordinated by three cysteine residues. A color version of the figure is available online.

A: classical radical SAM enzymes

B: PhdPh2

Figure 4.

The reaction catalyzed by classical radical SAM enzymes (A) and by PhdPh2 (B). Classical radical SAM enzymes generate a 5'-deoxyadenosyl radical, while PhdPh2 generates an ACP radical. The 5'-deoxyadenosyl radical normally undergoes hydrogen abstraction reactions, while the ACP radical generated by PhdPh2 likely undergoes an addition reaction to give the enzymatic product. A color version of the figure is available online.