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YBR246W Is Required for the Third Step of Diphthamide Biosynthesis

Xiaoyang Su,[†] Wei Chen,[‡] Wankyu Lee,[†,§] Hong Jiang,[†] Sheng Zhang,[‡] and Hening Lin*[,†]

[†]Department of Chemistry and Chemical Biology and [‡]Proteomics and Mass Spectrometry Core Facility, Cornell University, Ithaca, New York 14853, United States

Supporting Information

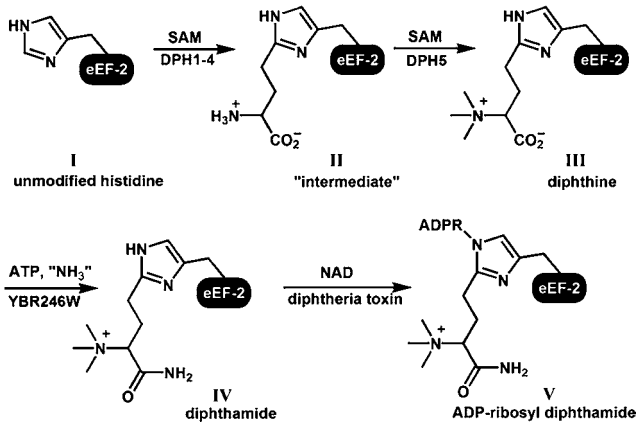
ABSTRACT: Diphthamide, the target of diphtheria toxin, is a post-translationally modified histidine residue that is found in archaeal and eukaryotic translation elongation factor 2. The biosynthesis and function of this modification has attracted the interest of many biochemists for decades. The biosynthesis has been known to proceed in three steps. Proteins required for the first and second steps have been identified, but the protein(s) required for the last step have remained elusive. Here we demonstrate that the YBR246W gene in yeast is required for the last step of diphthamide biosynthesis, as the deletion of YBR246W leads to the accumulation of diphthine, which is the enzymatic product of the second step of the biosynthesis. This discovery will provide important information leading to the complete elucidation of the full biosynthesis pathway of diphthamide.

Diphthamide is a post-translationally modified histidine found on archaeal and eukaryotic translational elongation factor 2 (eEF-2).[1−5] Diphthamide is the target of the diphtheria toxin (DT), which is an exotoxin produced by *Corynebacterium diphtheria*.[6] The toxin ADP-ribosylates diphthamide and therefore inactivates eEF-2 to stop ribosomal protein synthesis. While it is highly conserved from archaea to eukaryotes, the biological function of diphthamide remains poorly understood. The diphthamide modification has been reported to regulate translational fidelity in protein synthesis, and the lack of diphthamide results in increased −1 frameshift mutation.[7] However, no significant phenotype other than the toxin sensitivity has been linked to the lack of diphthamide modification in yeast.

The biosynthetic pathway of diphthamide has been elucidated in yeast and mammalian cells by biochemical and genetic studies.[8−16] There are three steps in the diphthamide biosynthesis, and five participating genes, *DPH1* through *DPH5*, have been identified (Scheme 1). The first step requires four proteins, Dph1−4, while the second step requires a single protein, Dph5. In contrast, the enzyme required for the last amidation step has remained unknown even three decades after the structure of diphthamide was revealed.

We recently reconstituted the first and second steps of diphthamide biosynthesis in vitro using proteins from a thermophilic archaea, *Pyrococcus horikoshii*.[17−19] Archaeal diphthamide biosynthesis differs from the eukaryotic system in that among the four proteins required for the first step (Dph1−Dph4), only one (Dph2) is present in archaea. *P. horikoshii* Dph2 (*Ph*Dph2) forms a homodimer in vitro and

Scheme 1. Biosynthesis Pathway of Diphthamide



uses a [4Fe−4S] cluster to generate a 3-amino-3-carboxypropyl radical to catalyze the first step of diphthamide biosynthesis.[17] On the basis of what was learned from *Ph*Dph2 and the fact that eukaryotic Dph1 and Dph2 are homologous and exist in a complex, we hypothesized that eukaryotic Dph1 and Dph2 form a heterodimer that is functionally equivalent to *Ph*Dph2 homodimer.[17] Dph3 and Dph4 most likely are required for the assembly of the [4Fe−4S] cluster or maintaining this cluster in the correct redox state.[17] Interestingly, one more protein, the product of yeast open reading frame (ORF) YBR246W, recently was reported to be required for the first step of diphthamide biosynthesis.[20] Because of our long-standing interest in the enzymology of diphthamide biosynthesis, this report triggered us to ask two questions: Is YBR246W really required for the first step? If so, what is the molecular role of YBR246W in the first step? Thus, we set out to validate the reported functional assignment of YBR246W. Our results show that YBR246 is actually required for the third step of diphthamide biosynthesis. This correct assignment for the function of YBR246W provides important information that will lead to the complete identification of the missing enzyme for the last step of diphthamide biosynthesis.

To confirm the involvement of YBR246W in diphthamide biosynthesis, we first performed an in vitro ADP-ribosylation reaction. His-tagged eEF-2 was overexpressed and purified from wild-type (WT) and DPH gene deletion strains. A rhodamine-labeled NAD compound (Rh-NAD) was used in the DT-catalyzed ADP-ribosylation reaction to visualize the product.[21]

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Without DT, eEF-2 was not labeled (Figure 1). When 0.1 μM DT was used, the eEF-2 from the WT yeast strain was clearly

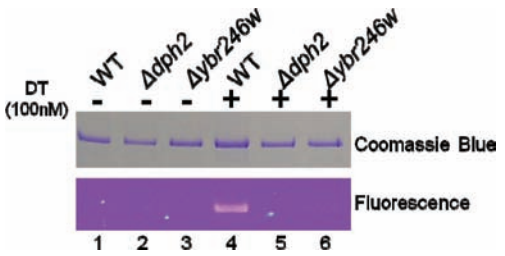

Figure 1. In vitro ADP-ribosylation using Rh-NAD. The upper panel displays a Coomassie blue-stained gel showing the eEF-2 proteins, and the lower panel shows the corresponding fluorescence labeling. The source strains from which the eEF-2 proteins were purified are labeled at the top. No DT was added in lanes 1–3, and 100 nM DT was added in lanes 4–6. Reactions were carried out at 30 °C for 20 min.

labeled, whereas the eEF-2 from Δ*dph2* or Δ*ybr246w* was not labeled (Figure 1). This result is consistent with the previous report that YBR246W is required for diphthamide biosynthesis.[20] Without YBR246W, diphthamide modification is impaired, and diphthamide cannot be ADP-ribosylated efficiently.

We then tested the requirement of YBR246W in diphthamide biosynthesis using an in vivo DT resistance assay similar to the one used to identify *DPH1–5* that are required for diphthamide biosynthesis.[9,12] WT, Δ*dph1*–Δ*dph5*, and Δ*ybr246w* strains were transformed with the pLMY101 plasmid,[12] which contains the catalytic domain of DT under the control of GAL1 promoter. When grown in glucose (Glc) medium, all strains were viable since DT expression was suppressed (Figure S1 in the Supporting Information). The Δ*dph3* strain showed a minor growth defect due to the participation of DPH3 in other biological processes.[15,22] When 2% galactose (Gal) was used as the carbon source, the WT strain did not grow because of the expression of the toxin, which ADP-ribosylates diphthamide on eEF-2. In contrast, *DPH1–5* deletion strains were viable because they do not synthesize diphthamide. However, the Δ*ybr246w* strain failed to grow on medium with 2% Gal (Figure 2A). These results show that unlike other *DPH* gene deletions, the YBR246W deletion does not confer DT resistance.

The DT sensitivity of Δ*ybr246w* was further examined by varying the Gal concentration in the medium to tune the expression level of DT. Raffinose (Raf) was used in combination with Gal to sustain the cell growth. Unlike Glc, which inhibits the GAL1 promoter transcription, Raf is a neutral carbon source that neither promotes nor inhibits GAL1-dependent expression. On plates with 2% Glc or 2% Raf, WT, Δ*dph2*, and Δ*ybr246w* strains were all viable. When 0.001% Gal was added, the WT strain showed a severe growth defect and Δ*ybr246w* grew normally. When 0.01% Gal was added, the growth of the WT was completely inhibited and the growth of Δ*ybr246w* was almost completely inhibited. Neither the WT nor the Δ*ybr246w* strain was able to grow when 0.1% Gal was present in the medium (Figure 2B). In contrast, the Δ*dph2* strain was able to grow even in the presence of 2% Gal. This result shows that Δ*ybr246w* is only slightly more resistant to DT than the WT is.

The in vitro ADP-ribosylation and in vivo DT sensitivity results were seemingly in conflict with each other. In other

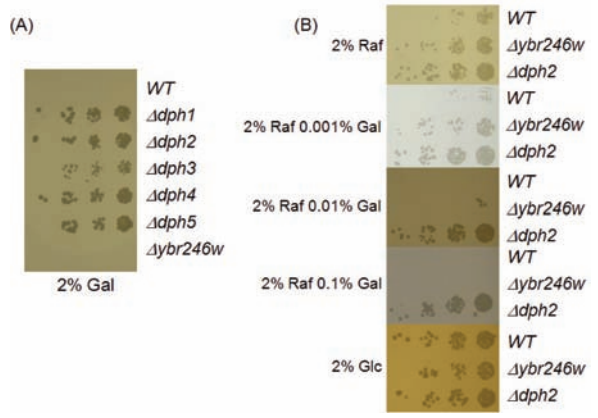

Figure 2. DT sensitivity assays of WT and deletion strains. (A) The strains were transformed with pLMY101, which encodes the catalytic fragment of DT, and then grown on 2% Gal medium. (B) WT, Δ*dph2*, and Δ*ybr246w* were grown on 2% Raf plus varying concentrations of Gal. The growth on 2% Glc served as a control.

words, how can DT kill the Δ*ybr246w* yeast cells if the eEF-2 cannot be ADP-ribosylated? Two possibilities were considered. First, without YBR246W, a small fraction of eEF-2 could still be fully modified while the majority remains unmodified. The small fraction of diphthamide modification would be enough to confer DT sensitivity. Second, the majority of eEF-2 actually could be modified but to a form that is different from diphthamide. To test the two possibilities, the in vitro eEF-2 labeling experiment was repeated with a much higher concentration of the toxin. If only a small fraction of eEF-2 were fully modified to diphthamide and could be ADP-ribosylated, then higher toxin concentrations would yield the same extent of labeling. However, the experimental result showed the opposite. The eEF-2 from the WT strain was labeled to similar levels at both low and high toxin concentrations, whereas the eEF-2 from Δ*ybr246w* was barely labeled when 0.1 μM DT was used but clearly labeled with 10 μM DT (Figure 3). In contrast, the eEF-2 from Δ*dph2* and

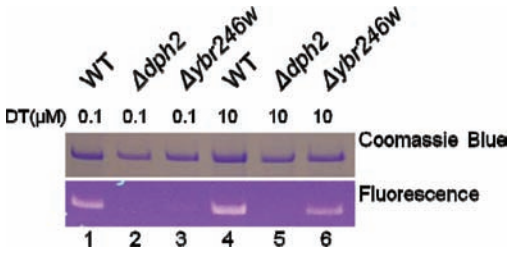

Figure 3. In vitro ADP-ribosylation assay at two different concentrations of DT. The upper panel displays the Coomassie blue-stained gel showing the eEF-2 proteins, and the lower panel shows the corresponding fluorescence labeling. The source strains from which the eEF-2 proteins were purified are labeled at the top. Reactions shown in lanes 1–3 contained 0.1 μM DT, and those shown in lanes 4–6 contained 10 μM DT. Reactions were carried out at 30 °C for 60 min.

Δ*dph5* was not labeled even when a high DT concentration was used (Figure 3 and Figure S2). These results indicate that the eEF-2 from Δ*ybr246w* can be ADP-ribosylated by DT, but less efficiently. Similar results were obtained by labeling of endogenous eEF-2 (Figure S3). The difference in the ability to be ADP-ribosylated supports the conclusion that eEF-2 from

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Δ*ybr246w* is different from the unmodified histidine (**I** in Figure 1), the intermediates in the biosynthesis (**II** in Figure 1), and the fully modified diphthamide (**IV** in Figure 1). Thus, the most logical possibility is that the eEF-2 from Δ*ybr246w* contains diphthine (**III**).

To confirm the presence of diphthine in the eEF-2 from Δ*ybr246w*, mass spectrometry (MS) studies were performed on eEF-2 purified from WT, Δ*dph2*, and Δ*ybr246w* yeast strains. The peptide (686-VNILDVTLHADAIHR-700) containing the unmodified histidine was found in all three eEF-2 samples (Figure S4 and S5). The diphthamide-containing peptide was found only in the eEF-2 from the WT strain, and the diphthine-containing peptide was found only in the eEF-2 from Δ*ybr246w* (Figure 4). The identities of diphthamide and

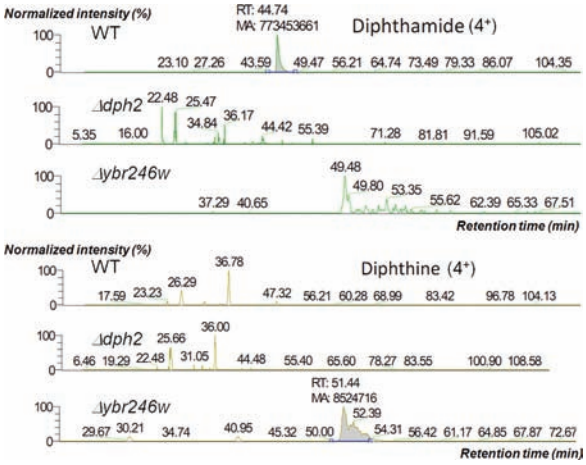

Figure 4. Extracted ion chromatograms of diphthamide and diphthine containing peptides from different strains. The peaks corresponding to diphthamide (calculated *m*/*z* 458.01567, extracted 458.01297−458.01755) and diphthine (calculated *m*/*z* 458.26181, extracted 458.25879−458.26337) containing peptides are highlighted in grey. The peptides carried 4 positive charges. The retention times (RT) and peak area by manual integration (MA) were shown above the highlighted peaks.

diphthine modifications on the peptides were supported by the MS/MS spectra (Figure 5). When the peptides are singly charged, the *m*/*z* for the peptide containing diphthamide is 0.984 unit smaller than the *m*/*z* for the peptide containing diphthine. Both diphthamide and diphthine undergo a neutral loss of a trimethylamino group during MS/MS, as reported earlier.[23] Because diphthine was present in Δ*ybr246w* yeast cells, we concluded that YBR246W is not required for the first step of diphthamide biosynthesis. The accumulation of diphthine in Δ*ybr246w* but not in the WT suggests that YBR246W is required for the last amidation step of diphthamide biosynthesis.

We previously reported that in *P. horikoshii* EF2 (*Ph*EF-2), diphthine is not stable and readily eliminates the trimethylamino group and a proton in a reaction that is similar to Hofmann−Cope elimination.[19] This elimination is similar to but different from the neutral loss of the trimethylamino group we observed for yeast diphthine during MS/MS. The elimination occurs before MS, while the neutral loss occurs during MS/MS. Two possible mechanisms for the elimination reaction of *Ph*EF-2 diphthine were proposed.[19] One mechanism uses an external base to attack the proton on the β-carbon, and the other mechanism uses the carboxyl group as

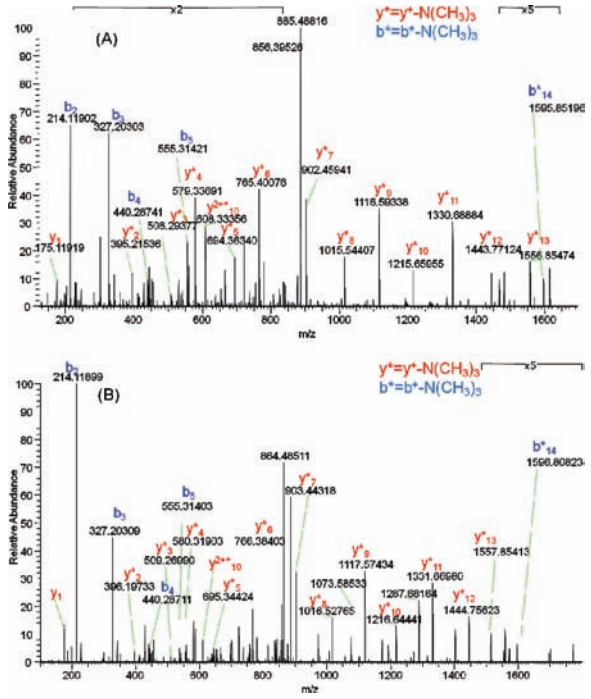

Figure 5. MS/MS spectra of diphthamide (A, parental ion *m*/*z* 915.02325) and diphthine (B, parental ion *m*/*z* 915.51520) containing peptides. A neutral loss of the trimethyl amino group was observed in both spectra and labeled b* and y*.

the intramolecular base to deprotonate the β-carbon. Since the elimination reaction is species-dependent, it is possible that the actual base is a neighboring residue that is present in *Ph*EF-2 but not in yeast eEF-2.

The previous genetics study by Carette et al.[20] provided the crucial information that YBR246W is involved in diphthamide biosynthesis. However, our data presented here demonstrate that their biochemical function assignment is incorrect. The evidence used by Carette et al. to support their conclusion that YBR246W is required for the first step was that eEF-2 from Δ*ybr246w* cannot be ADP-ribosylated and contained unmodified histidine residue. Our results demonstrate that although both observations can be repeated, they are only partially true. We have shown that eEF-2 from Δ*ybr246w* cannot be labeled when a low concentration of DT is used but can be labeled when a higher DT concentration is used. This labeling pattern is different from eEF-2 isolated from the Δ*dph2* strain, which cannot be labeled even at higher DT concentrations. Our study has also shown that unmodified eEF-2 exists even in the WT strain, consistent with a previous report.[3] Therefore, the presence of unmodified histidine in Δ*ybr246w* does not indicate that the first step modification is impaired. Instead, it may suggest that the first step of diphthamide biosynthesis is rate-limiting.

The accumulation of diphthine in the Δ*ybr246w* strain but not in other DPH gene deletion strains or the WT strain demonstrates that YBR246W is required for the third step of diphthamide biosynthesis. Whether YBR246W alone is sufficient for the amidation step is unknown. It is possible that other proteins are also required. YBR246W contains WD40 repeats, which suggests that it may be a scaffold protein for the amidation step rather than a catalytic subunit.[24,25] YBR246W may be used to pull down other proteins required for the last step. Previous yeast genetic studies identified five

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DPH genes (DPH1−5), but the YBR246W gene was not revealed. The reason is that under the selection conditions previously used, Δ*ybr246w* was not viable to be selected, as we have shown in Figure 2. We have observed that at a lower toxin induction level, the Δ*ybr246w* strain is able to grow while the WT strain cannot. These findings may facilitate the identification of other genes involved in the amidation step. A recent report showed that YBR246W also functions in the retromer-mediated endosomal recycling pathway that is important for recycling amino acid transporters back to the plasma membrane.[25] The fact that YBR246W functions in two apparently different biological pathways suggests an interesting possibility that YBR246W, as a possible scaffold protein, may coordinate nutrient availability (via recycling of amino acid transporters) and translation (via diphthamide biosynthesis). This may provide important clues for understanding the function of diphthamide in protein biosynthesis, which has been almost completely unknown for more than three decades.

■ 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.

■ AUTHOR INFORMATION

Corresponding Author

hl379@cornell.edu

Present Address

[§]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

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