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YBR246W is required for the third step of diphthamide biosynthesis

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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, however, the protein(s) required for the last step 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 to finally complete 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, namely *DPH1* – *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.

Recently, we have reconstituted the first and second step of diphthamide biosynthesis *in vitro* using proteins from a thermophilic archaea, *P. horikoshii*.^{17–19} Archaeal diphthamide biosynthesis differs from the eukaryotic system in that among the four proteins (Dph1–Dph4) required for the first step, only one (Dph2) is present in archaea. *P. horikoshii* Dph2 (*PhDph2*) forms a homodimer *in vitro*, and uses a [4Fe-4S] cluster to generate a 3-amino-3-carboxypropyl radical to catalyze the first step of diphthamide biosynthesis.¹⁷ Based on

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

what was learned from *PhDph2* and that eukaryotic Dph1 and Dph2 are homologous and exist in a complex, we hypothesized that eukaryotic Dph1 and Dph2 forms a heterodimer and is functionally equivalent to *PhDph2* homodimer.¹⁷ Dph3 and Dph4 most likely are required for the assembly of the [4Fe-4S] cluster or maintaining the [4Fe-4S] 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 over-expressed and purified from wild-type (WT) and *DPH* gene deletion strains. A rhodamine-labeled NAD compound (Rh-NAD) was used in DT-catalyzed ADP-ribosylation reaction to visualize the product.²¹ Without DT, eEF-2 was not labeled (Figure 1). When 0.1 μ M DT was used, the eEF-2 from WT yeast strain was clearly labeled. The eEF-2 from Δ *dph2* or Δ *ybr246w* was not labeled (Figure 1). This result was consistent with the previous report that YBR246W is required for diphthamide biosynthesis.²⁰ Without YBR246W, diphthamide modification is impaired and cannot be ADP-ribosylated efficiently.

We then tested the requirement of YBR246W in diphthamide biosynthesis using an *in vivo* DT resistance assay. This assay was similar to the one used to identify *DPH1-DPH5* 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). The Δ *dph3* strain showed 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 wild-type strain did not grow due to 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). The results showed 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 does not promote nor inhibit 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, WT strain showed severe growth defect and Δ *ybr246w* grew normally. When 0.01% Gal was added, the growth of 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* was only slightly more resistant to DT than WT.

The *in vitro* ADP-ribosylation results and the *in vivo* DT sensitivity results were seemingly in conflict with each other. In other 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 is still fully modified while the majority remains unmodified. The small fraction of diphthamide modification is enough to confer DT sensitivity. Second, the majority of eEF-2 is actually 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 is fully modified to diphthamide and can 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. The eEF-2 from the $\Delta ybr246w$ was barely labeled when 0.1 μM DT was used, but was labeled clearly when 10 μM of DT was used (Figure 3). In contrast, the eEF-2 from $\Delta dph2$ and $\Delta dph5$ was not labeled even when high DT concentration was used (Figure 3 and S2). These results indicate that the eEF-2 from $\Delta ybr246w$ 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 that eEF-2 from $\Delta ybr246w$ is different from the unmodified histidine (I in Figure 1), the intermediates (II in Figure 1) in the biosynthesis, or the fully modified diphthamide (IV in Figure 1). Thus the most logic possibility is that the eEF-2 from $\Delta ybr246w$ contains diphthine (III).

To confirm the presence of diphthine in the eEF-2 from $\Delta ybr246w$, mass spectrometry (MS) studies were performed on eEF-2 purified from WT, $\Delta dph2$ and $\Delta ybr246w$ 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 only found in the eEF-2 from WT strain, and the diphthine-containing peptide was found only in the eEF-2 from $\Delta ybr246w$ (Figure 4). The identities of diphthamide and diphthine modifications were supported by the MS/MS spectra (Figure 5). When singly charged, the m/z for the peptide containing diphthamide is one Dalton smaller than the m/z for the peptide containing diphthine. Both diphthamide and diphthine undergo a neutral loss of trimethylamino group during MS/MS, which was reported earlier.²³ Because diphthine was present in $\Delta ybr246w$ yeast cells, we concluded that YBR246W is not required for the first step of diphthamide biosynthesis. The accumulation of diphthine in $\Delta ybr246w$ but not in WT suggests that YBR246W is required for the last amidation step of diphthamide biosynthesis.

We have previously reported that in *P. horikoshii* EF2 (*PhEF*-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 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 *PhEF*-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 the intra-molecular 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 *PhEF*-2 but is not present in yeast eEF-2.

Previous genetics study provided crucial information that YBR246W is involved in diphthamide biosynthesis.²⁰ However, our data presented here demonstrated that the biochemical function assignment was incorrect. The evidence used by Carette et al. to support that YBR246W was required for the first step was that eEF-2 from $\Delta ybr246w$ cannot be ADP-ribosylated and contained unmodified histidine residue. Our results demonstrated that although both observations can be repeated, they are only partially true. We have shown that eEF-2 from $\Delta ybr246w$ cannot be labeled when low concentration of DT was used, but can be labeled when higher concentration of DT was used. This labeling pattern is different

from eEF-2 isolated from *Δdph2* strain, which cannot be labeled even when higher concentration of DT was used. Our study also showed that unmodified eEF-2 exists even in the WT strain, consistent with a previous report.³ So the presence of unmodified histidine in *Δybr246w* does not support 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 *Δybr246w* strain but not in other DPH gene deletion strains or WT strain demonstrated 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 DPH genes (DPH1-5), but YBR246W gene was not revealed. The reason is that under the selection condition previously used, *Δybr246w* was not viable to be selected, as we have shown in Figure 2. We observed that at a lower toxin induction level, the *Δybr246w* strain was able to grow while the WT strain was not. 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 acids 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 acids transporters) and translation (via diphthamide biosynthesis). This may provide important clues to understand the function of diphthamide in protein biosynthesis, which has been almost completely unknown for more than three decades.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

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

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 were grown on 2% Gal medium. (B) WT, $\Delta dph2$ and $\Delta ybr246w$ were grown on 2% Raf plus varying concentrations of Gal. The growth on 2% Glc was shown as a control.

Figure 3.

In vitro ADP-ribosylation assay with two different concentrations of DT. The upper panel was the Coomassie blue-stained gel showing the eEF-2 proteins and the lower panel showed the corresponding fluorescence labeling. The source strains from which the eEF-2 proteins were purified were labeled above. Reactions shown in lane 1–3 contained 0.1 μ M of DT and those shown in lane 4–6 contained 10 μ M of DT. Reactions were carried out at 30°C for 60 min.

Figure 4.

Extracted ion chromatograms of diphthamide and diphthine from different strains. The peaks corresponding to diphthamide and diphthine containing peptides were highlighted in grey. The peptides carried 4 positive charges. The retention time (RT) and peak area integration (MA) were shown above the highlighted peaks.

Figure 5. MS/MS spectra of peptides containing diphthamide (A) and diphthine (B). A neutral loss of the trimethyl amino group was observed in both spectra.

Scheme 1.
Biosynthesis pathway of diphthamide.