Supporting information

Dph3 is an electron donor for Dph1-Dph2 in the first step of eukaryotic diphthamide biosynthesis
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Materials and methods

Cloning, expression of Dph1-Dph2
Yeast Dph1 and Dph2 were amplified from yeast genomic DNA, which was extracted from BY4741 using Pierce Yeast DNA Extraction Kit. The primers used for Dph1 were XS022 (5'-agtcaagaatcTATGAGTGGGCTCTACAGAATC-3') and HL002 (5'-agtcaagtcacCTATTCAATCGCATGTTCGG-3') and the primers used for Dph2 were XS035 (5'-agtcaGATATGTA caccatcaccatcaccatGAAGTTGCCGCTTT-3') and XS004 (5'-agtcaGGTACC tcatggtttctttttcatagc-3'). Dph2 gene was inserted into pETduet-1 vector first, followed by the insertion of Dph1. The Dph1-Dph2 gene from pETduet-1 was amplified using primers XS022 and XS004 and transferred to the pET28b vector. The plasmids pET28duet-1 Dph1-Dph2 and pET28b Dph1/Dph2 were used to transform BL21(DE3) pRARE2 strain respectively. Plasmid pDB1282, which contains the Azotobacter vinelandii isc operon under control of an arabino-inducible promoter, was cotransformed with Dph1/Dph2 plasmid. Cells were grown in 2 liters LB medium with 100 μg/ml ampicillin, 20 μg/mL chloramphenicol and 50 μg/mL kanamycin at 37°C and 200 rpm. At optical density (OD_600) of 0.3, solid arabinose was added to each flask at a final concentration of 0.05 % (w/v). When the OD_600 reached to 0.6, the cultures were cooled down to 18°C and supplemented with FeCl_3, Fe(NH_4)_2(SO_4)_2 and L-cysteine to final concentrations of 50 μM, 50 μM and 400 μM, respectively. Protein expression was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), at which point the culture flasks were sealed to limit the amount of oxygen in the system. Cells were incubated in a shaker at 18°C and 200 rpm for 20 h before harvested.

Anaerobic purification of Dph1-Dph2 complex.

Purification of Dph1-Dph2 was performed in an anaerobic chamber (Coy Laboratory Products). Cell pellet from 2 liter culture was suspended in 30 mL lysis buffer (500 mM NaCl, 10 mM MgCl_2, 5 mM imidazole, 1 mM DTT, and 200 mM Tris-HCl at pH 7.4). Lysozyme (45 mg, 0.3% w/v) and nuclease (3 μL, 25 U/ml of lysis buffer, Thermo) were added and incubated at 25 °C for 1 h. The mixture was then frozen with liquid nitrogen and then thawed at 25 °C. Cell debris was removed by centrifugation at 20,000g (Beckman Coulter Avanti J-E) for 30 min. The supernatant was incubated for 1 h with 1.2 ml Ni-NTA resin (Qiagen) pre-equilibrated with the lysis buffer. The Ni-NTA resin was loaded onto a polypropylene column and washed with 20 ml lysis buffer, followed by 20 ml of 30 mM imidazole in lysis buffer. Dph1-Dph2 was eluted from the column with elution buffers (100 mM, 150 mM and 200 mM imidazole in the lysis buffer, 1.5 mL each). The brown-colored elution fractions were buffer-exchanged to 150 mM NaCl, 1 mM DTT and 200 mM Tris-HCl at pH 7.4 and 5% glycerol using a Bio-Rad 10-DG desalting column. The
purified proteins were concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore). Protein concentration was determined by Bradford assay.

**Size-Exclusion Chromatography-Coupled Multiangle Light Scattering (SEC-MALS).** Purified Dph1-Dph2 protein (~10 μg/μL) was applied to SEC using a Yarra SEC 4000 column (Phenomenex Inc.) equilibrated in MALS buffer (200 mM Tris-HCl pH 7.4, 150 mM NaCl). The SEC was coupled to a static 18-angle light scattering detector (DAWN HELEOS-II) and a refractive index detector (Optilab T-rEX) (Wyatt Technology). Data were collected every second at a flow rate of 1 mL/min. Data analysis was carried out using the program ASTRA, giving the molar mass and mass distribution (polydispersity) of the sample. Monomeric BSA (Sigma) was used for normalization of the light scattering detectors and data quality control.

**UV-Vis spectroscopy of Dph1-Dph2** Dph1-Dph2 (50 μM) was prepared anaerobically in 150 mM NaCl and 200 mM Tris-HCl at pH 7.4 and 5% glycerol. The dithionite treated sample was allowed to incubate for 10 min after adding the reducing agent at a final concentration of 0.5 mM. The samples were sealed in a quartz cell (100 μl each) before being taken out of the anaerobic chamber. The UV-Vis spectra were obtained on a Cary 50 Bio UV-Vis spectrophotometer (Varian), scanning from 200 nm to 800 nm. The baseline was corrected with the buffer used to prepare the samples.

**EPR spectroscopy of Dph1-Dph2** To the purified Dph1-Dph2 (700 μM), dithionite was added to a final concentration of 1 mM in an anaerobic chamber. The mixture was transferred to EPR tubes, frozen in liquid N₂ and sealed for EPR analysis. EPR spectra were recorded at ACERT on a Bruker ElexSys E500 EPR spectrometer at a frequency of 9.34 GHz in quartz tubes with internal diameters of 4 mm. EPR measurement was carried out at 12 K using an ESR910 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.

**Expression and purification of yeast eEF2** The Saccharomyces cereisiae eEF2 was expressed and purified essentially as described before but with the following modification. After harvesting and resuspending the cells in 20 mM Tris-HCl buffer (pH 8.0) containing 400 mM (NH₄)₂SO₄, 10 mM MgCl₂, and 1 mM protease inhibitor PMSF, cells were lysed using glass beads with bead beater (Biospec).

**Analyzing reaction product from Dph1-Dph2 with high-performance liquid chromatography** Under anaerobic condition, reactions were assembled with 60 μM Dph1-Dph2, 30 μM SAM, 10 mM dithionite, 150 mM NaCl, 1 mM DTT and 200 mM Tris-HCl at pH 7.4 in a final volume of 30 μl. The mixture was incubated at 25 °C for 60 min. The reactions were carried out with or without (30 μM) eEF2. Negative control reactions without SAM, Dph1-Dph2, or dithionite were also carried out. Then the reaction was quenched with 30 μl of 10% TFA, followed by centrifugation to separate the precipitated proteins and the supernatant. The supernatant was
analyzed by high-performance liquid chromatography (Shimadzu) on a C18 column (Kinetex) monitored at 254 nm absorbance, using a linear gradient from 0 to 40% buffer B over 15 min at a flow rate of 0.5 ml/min (solvent A: 0.1% aqueous TFA and solvent B: 0.1% TFA in acetonitrile).

Anaerobic reconstitution of Dph1-Dph2 activity
The reaction mixture was assembled in an anaerobic chamber. The reactions contain 40 μM Dph1-Dph2, 7 μM eEF-2 and 5 mM dithionite in 150 mM NaCl, 1 mM DTT and 200 mM Tris-HCl at pH 7.4. The reaction vials were sealed before being taken out of the anaerobic chamber. 14C-SAM (18 μM; 55 mCi/mmol, American Radiolabeled Chemicals Inc.) was injected into each reaction vial to start the reaction. The reaction mixtures were vortexed briefly to mix and incubated at 25 °C for 60 min. The reactions were stopped by adding protein loading dye 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).

Cloning, expression and purification of Dph3
Yeast Dph3 was amplified from yeast genomic DNA, which was extracted from BY4741 using Pierce Yeast DNA Extraction Kit. The primers used for Dph3 were XS024 (5'-agtcagGA TA TCgatgtcaacatatgacgaaatc-3') and JT008 (5'-AGTCAGCTCGAGTTA atggtatggattgttagtgGCGACGCGCCGCAATAGG-3'). Dph3 was inserted into pCDFduet vector. The plasmid was used to transform BL21(DE3) pRARE2 strain. Cells were grown in 2 liters LB medium at 37°C and 200 rpm. When the OD600 reached to 0.6, the cultures were cooled down to 18°C and supplemented with (or without) Fe(NH4)2(SO4)2 to a final concentration of 70 μM. Protein expression was induced by 0.1 mM IPTG. Cells were harvested after incubation at 18°C for 20 h. Cell pellet was resuspended in 30 ml of buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl and 0.5 mM phenylmethylsulfonyl fluoride. Cells were lysed using the EmulsiFlex-C3 cell disruptor (Avestin, Inc. Canada). Dph3 was purified through Ni-NTA affinity chromatography following the same protocol as that used for the purification of Dph1-Dph2. The eluted imidazole fractions were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, 1 mM DTT, and 5% glycerol. Purified protein was concentrated using Amicon Ultra-4 centrifugal filter devices.

Ultraviolet–visible spectroscopy of Dph3
The UV-Vis spectra of Dph3 (80 μM) were obtained on an UV-Vis spectrophotometer, scanning from 200 nm to 800 nm. The baseline was corrected with the buffer used to prepare the samples. Dithionite was added to the sample at a final concentration of 0.5 mM. After obtaining the UV-Vis spectrum, the sample was then exposed to air to re-oxidize Dph3. The spectrum was then taken again.

Cloning, expression and purification of NorW
NorW gene was amplified from the genomic DNA of E.coli strain TOP10. The primers used were XS055 (5'-agtcagCATATGagtaagcatgttcatc-3') and XS056 (5'-agtcagCTCGAG ctacatggaatcttc-3'). NorW was inserted into pET28a vector. The plasmid was used to transform BL21(DE3) pRARE2 strain. Cells were grown in 2 liters LB medium at 37°C and 200
rpm. It took 4-5 hours for the OD$_{600}$ to reach 0.5 after inoculation with the overnight culture. Then the culturing temperature was changed to 18°C, and the protein expression was induced by 0.1 mM IPTG. Cells were harvested after incubation at 18°C for 20 h. The protein was purified using a HisTrap column (GE Healthcare). Protein concentration was determined by Bradford assay.

**Reduction of Dph3 with NorW and NADH**

The reaction was monitored on a Cary 50 Bio UV-Vis spectrophotometer (Varian) at 488 nm. Dph3 was used at the concentration of 87 μM. NorW was added to a final concentration of 1 μM. NADH was used at a final concentration of 2 mM.

**Expression and Purification of Zn$^{2+}$-bound Dph3**

The same strain as Fe bound Dph3 was used. Cells were grown in M9 minimal medium supplemented with 0.2% (w/v) glucose, 2 mM MgSO$_4$ and 0.1 mM CaCl$_2$ at 37°C and 200 rpm. When the OD$_{600}$ reached to 0.8, the cultures were cooled down to 18°C and supplemented with ZnCl$_2$ to a final concentration of 70 μM. Protein expression was induced by 0.1 mM IPTG. Cells were harvested after incubation at 18°C for 20 h. Cells were collected and Zn-bound Dph3 was purified through Ni-NTA affinity chromatography following the same protocol as that used for the purification of Fe-bound Dph3.

**Anaerobic reconstitution of the first step of yeast diphthamide biosynthesis using Dph1-3**

The reaction mixture was assembled in the anaerobic chamber under strictly anaerobic conditions. Aerobically purified proteins, Dph3, NorW and eEF-2 were degassed by schlenk line. The reaction component, 40 μM Dph1-Dph2, 30 μM Dph3 or Zn$^{2+}$ bound Dph3, 7 μM eEF-2, 60 μM NorW and 400 μM NADH were added in the buffer of 150 mM NaCl, 1 mM DTT and 200 mM Tris-HCl at pH 7.4. Negative control reactions without NADH or NorW were also carried out. The reaction vials were sealed before being taken out of the anaerobic chamber. $^{14}$C-SAM (2 μl, final concentration 18 μM) was injected into each reaction vial to start the reaction. The reaction mixture was vortexed briefly to mix and incubated at 30°C for 60 min. The reaction was stopped by adding protein loading dye and subsequently heating at 95°C for 5 min, and then resolved by 4-20% SDS–polyacrylamide gel electrophoresis. The dried gel was exposed to a PhosphorImaging screen and the radioactivity was detected using a STORM 860 PhosphorImager.

**Expression and purification of Dph4**

Yeast Dph4 was amplified from yeast genomic DNA, which was extracted by BY4741 using Pierce Yeast DNA Extraction Kit. The primers used for Dph4 were XS023 (5’-agtcatGGATCCcatgtcttggtgaattcgttaa-3’) and XS003 (5’-agtcatGTCGACtatgtgcatctct-3’). Dph4 gene was inserted into pDEST 566 vector. The plasmid was used to transform BL21(DE3) pRARE2 strain. Cells were grown in 2 liters LB medium at 37°C and 200 rpm. When the OD$_{600}$ reached to 0.6, the cultures were cooled down to 18°C and supplemented with FeCl$_3$ to a final concentration of 200 μM. Protein expression was induced by 0.1 mM IPTG. Cells were harvested after incubation at 18°C for 20 h. Cell pellet was resuspended in 30 ml of buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl and 0.5 mM phenylmethylsulfonyl fluoride. Cells were lysed using the EmulsiFlex-C3 cell disruptor (Avestin, Inc. Canada). His$_6$-MBP tagged Dph4 was purified through Ni-NTA affinity chromatography following the same protocol as that
used for the purification of Dph1-Dph2. The eluted imidazole fractions were collected and dialyzed together with His tagged TEV against 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, 1 mM DTT, and 5% glycerol. The protein mixture was applied to Ni-NTA affinity chromatography again to get rid of His6-MBP and His tagged TEV. Dph4 was eluted with 30 mM imidazole in lysis buffer. The purified protein was concentrated using Amicon Ultra-4 centrifugal filter devices.

**Anaerobic reconstitution of PhDph2 activity using carboxy-14C-SAM**

PhDph2 and PhEF2 were expressed and purified as described before. The reconstitution was performed following the same protocol as that used for Dph1-3.

![ SEC-MALS/UV analysis of Dph1/Dph2. SEC profile monitored by absorbance at 280 nm is shown as black line. The calculated MW values are plotted. Dph1 and Dph2 exist as a heterodimer (110 kDa). The concentration of the sample was 10 mg/mL. ]

**Figure S1.** SEC-MALS/UV analysis of Dph1/Dph2. SEC profile monitored by absorbance at 280 nm is shown as black line. The calculated MW values are plotted. Dph1 and Dph2 exist as a heterodimer (110 kDa). The concentration of the sample was 10 mg/mL.

![ Coomassie blue stained SDS-PAGE showing purified proteins. A) eEF2, Dph1/Dph2, Fe-Dph3, NorW and Zn2+-bound Dph3. B) MBP-Dph4 before and after TEV treatment. ]

**Figure S2.** Coomassie blue stained SDS-PAGE showing purified proteins. A) eEF2, Dph1/Dph2, Fe-Dph3, NorW and Zn2+-bound Dph3. B) MBP-Dph4 before and after TEV treatment.
Figure S3. *In vitro* reconstitution of PhDph2 activity using carboxy-$^{14}$C-SAM. The top panel shows the Coomassie-blue-stained gel; the bottom panel shows the autoradiography. Both the reactions contained PhEF2 and PhDph2. The presence of other reagents is indicated below each lane.

References:

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