Supporting Information

Dph3 enables aerobic diphthamide biosynthesis by donating one iron atom to transform a [3Fe-4S] to a [4Fe-4S] cluster in Dph1-Dph2

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Material and methods

Anaerobic expression and purification of Dph1-Dph2. Dph1-Dph2 was expressed and purified following published methods.[1]

Aerobic expression and purification of Dph1-Dph2. Dph1-Dph2 was expressed and purified following published method with modifications, as described below.[1] During the expression of Dph1-Dph2, flasks are not sealed to limit the concentration of oxygen in the flask. Cell pellet from 2L culture was suspended in 20 mL lysis buffer containing 50 mM Tris (pH=8.0), 500 mM NaCl and 5% glycerol. The suspension was lysed using the EmulsiFlex-C3 cell disruptor (Avestin, Inc.). Cell debris was removed

by centrifugation at 18,000g in Sorvall Lynx 4000 Centrifuge (Thermo Scientific) for 30 min. The supernatant was incubated for 30 min with 1 ml Ni-NTA resin (Qiagen) pre-equilibrated with the lysis buffer in 4 °C. The Ni-NTA resin was loaded onto a polypropylene column and washed with 50 ml lysis buffer, followed by 50 ml of 15 mM imidazole in lysis buffer. Protein was eluted from the column with 100 mM imidazole in lysis buffer until the elution has no color. The colored fraction was concentrated into 2 mL then loaded into AKTA PURE system. Protein was passed through Highload™ 16/60 Superdex™ 200 pg column with desalting buffer containing 200 mM Tris (pH 8.0), 150 mM NaCl and 10% glycerol with the following method. The column was equilibrated with 120 mL desalting buffer with flow rate 1 mL/min. Sample loop was washed with 10 mL desalting buffer with flow rate 1 mL/min. Protein fraction was eluted with 150 mL desalting buffer with flow rate 1 mL/min. The protein fraction was collected 2 mL/ tube with the first 24 mL discarded. UV absorption was monitored at 280 nm, 410 nm and 488 nm. The fraction with high 410 nm absorption was checked with 12% SDS page protein gel, collected, and concentrated to around 600 μM final concentration.

Purification of Dph3. Yeast Dph3 was amplified from genomic DNA using primer YZ250_scDph3_NcoI_5' (agtcagCCATGGGCTCAACATATGACGAAATCGAA) and YZ004_C-His_ dph3_XhoI_3' ACCAACTCGAGTTAatgatgatgatgatgatgGGCAGCAGCGGCAATAG). Dph3 was inserted into pET28a vector. The plasmid was used to transform BL21(DE3) strain. A single colony was inoculated into 20 mL LB medium at 37 °C for overnight culture.

The overnight culture was inoculated into 2 L M9 medium (Ameresco). When OD_{600} reaches 0.6, the media was cooled to 16 °C and $FeCl_3$ was added to a final concentration of 100 μM. Protein expression was induced with 0.15 mM IPTG. After 20 hrs, cells were harvested and Dph3 was purified following a published method.[1] The ^{57}Fe enriched Dph3 was obtained by addition of ^{57}Fe powder dissolved in HCl and adjust pH to 5-6 instead of $FeCl_3$ in the culture of the bacteria.

Purification of EF2. Yeast pellet overexpressing EF2 was obtained from the fermentation service done by Bioexpression and Fermentation Facility at University of Georgia. The protein was purified following a published method.[2]

Anaerobic reconstitution of the first step of yeast diphthamide biosynthesis with Dph3-Fe^{2+}. Truncated Cbr1 protein was expressed and purified as previously described.[3] Aerobically purified Dph3, Cbr1 and eEF2 were degassed using a Schlenk line. The Dph1-Dph2 used in the anaerobic reconstitution was purified anaerobically. The reconstitution reactions were set up in an anaerobic chamber. The proteins and reagents were added in the following sequence: Dph3 (10 μM), Cbr1 (2 μM), Dph1-Dph2 (2 μM), eEF2 (2 μM) and NADH (2 mM), or Dph1-Dph2 (2 μM), eEF2 (2 μM) and dithionite (10 mM). The reaction vials were sealed before being taken out of the anaerobic chamber. Carboxy-^{14}C-SAM (ARC 0343-50, 18 μM) was injected into each reaction vial to initiate the reaction. The reaction mixtures were mixed by brief vortexing and incubated at room temperature for indicated time. The reactions were stopped by adding protein loading dye and subsequently heating at 95 °C for 5 min, and then resolved by 12% SDS-PAGE. The dried gel was exposed to a Phosphor Imaging

screen and scanned using a Typhoon FLA 7000 (GE Healthcare Life Sciences).

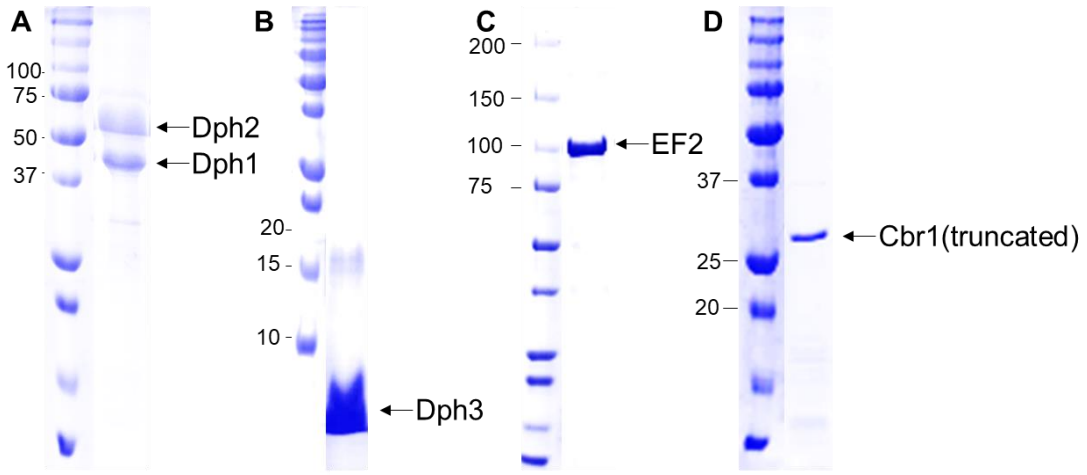
Aerobic reconstitution of the first step of diphthamide biosynthesis. The proteins used in the aerobic reconstitution were all purified aerobically including Dph1-Dph2. The proteins and reagents were added to the reaction buffer in the following sequence: Dph3 (10 μM), Cbr1 (2 μM), Dph1-Dph2 (2 μM), eEF2 (2 μM), Carboxy-^{14}C-SAM (ARC 0343-50, 18 μM) and NADH (2 mM), or Dph1-Dph2 (2 μM), eEF2 (2 μM), Carboxy-^{14}C-SAM (ARC 0343-50, 18 μM) and dithionite (10 mM). The reaction buffer contains 150 mM NaCl, 200 mM Tris-HCl at pH 8.0 and 10% glycerol. The reconstitution was performed in aerobic conditions including all the buffer preparation. Sodium citrate was added directly into buffer to a final concentration of 2 mM in reaction with citrate. The reaction was performed at room temperature for 30 mins. The reactions were stopped by adding protein loading dye and subsequently heating at 95 °C for 5 min, and then resolved by 12% SDS-PAGE. The dried gel was exposed to a Phosphor Imaging screen and scanned using a Typhoon FLA 7000 (GE Healthcare Life Sciences).

Electron paramagnetic resonance (EPR) spectroscopy. EPR was performed with published method with the following modification.[4] Dph1-Dph2 (200 μM), eEF2 (100 μM), and SAM (1 mM) were mixed with or without Dithionite (100 mM) in 100 μL total volume with desalting buffer containing 200 mM Tris (pH 8.0), 150 mM NaCl and 10% glycerol. The EPR tubes were immediately frozen in liquid nitrogen. EPR tubes were kept in liquid nitrogen Dewar before the EPR measurement.

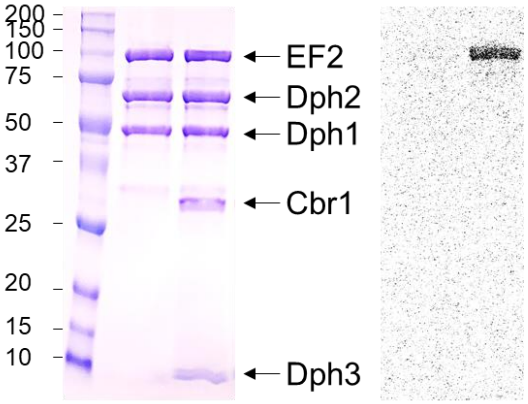
Rapid freeze quench. RFQ experiments were performed with a Bio-Logic SFM300 with two glass syringes. One syringe was filled with 400 μM Dph1-Dph2, 2 mM Dph3,

200 μM EF2, 100 μM Cbr1 and 2 mM SAM in buffer containing 200 mM Tris-HCl, pH 8.0, 150 mM NaCl and 10% glycerol. Another syringe was filled with 20 mM NADH in buffer containing 200 mM Tris-HCl, pH 8.0, 150 mM NaCl and 10% glycerol. The solutions were loaded into the RFQ and mixed at 1:1(v/v) ratio (200 μl total, 100 μl per sample) and collected at selected time points. The reaction mixture was injected into a funnel coupled to an EPR tube and filled with liquid ethane (~-170 °C). The frozen samples were packed using a stainless-steel rod. The tubes were stored in liquid nitrogen tank before spectroscopic characterization.

Supplementary Figures and Tables



Supplementary Figure 1. Purification of Dph1-Dph2 (A), Dph3 (B), EF2 (C) and truncated Cbr1 (D). Ladder size and protein name were labeled



Supplementary Figure 2. Representative full gel image of *in vitro* reconstitution of the first step of biosynthesis of diphthamide. Coomassie blue stained 12% SDS page (left). Protein name was labeled. Autoradiograph image (right).

Supplementary Table 1. Quantification of Fe-S species in Fig 2C&2D

	Aerobically purified (Fig. 2C)	Anaerobically purified (Fig. 2D)
$[3Fe\text{-}4S]^{1+}$	58.5%	0.3%
$[4Fe\text{-}4S]^{1+}$	41.5%	99.7

Supplementary Table 2. Bacteria strain used.

Strain	Plasmid	Source
HL874E	pET28a Dph1-Dph2&pDB1282	Dong, M. *et al*[1]
HL1822E	pET28a truncated Cbr1	Lin, Z. *et al*[3]
HL1821E	pET28a Dph3-His	This study

Reference

1. Dong, M.; Su, X.; Dzikovski, B.; Dando, E. E.; Zhu, X.; Du, J.; Freed, J. H.; Lin, H., Dph3 is an electron donor for Dph1-Dph2 in the first step of eukaryotic diphthamide biosynthesis. *J Am Chem Soc* **2014,** *136* (5), 1754-1757.
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3. Lin, Z.; Dong, M.; Zhang, Y.; Lee, E. A.; Lin, H., Cbr1 is a Dph3 reductase required for the tRNA wobble uridine modification. *Nat Chem Biol* **2016,** *12* (12), 995-997.
4. Dong, M.; Kathiresan, V.; Fenwick, M. K.; Torelli, A. T.; Zhang, Y.; Caranto, J. D.; Dzikovski, B.; Sharma, A.; Lancaster, K. M.; Freed, J. H.; Ealick, S. E.; Hoffman, B. M.; Lin, H. N., Organometallic and radical intermediates reveal mechanism of diphthamide biosynthesis. *Science* **2018,** *359* (6381), 1247-1250.