Supporting Information

Bioinformatic and biochemical characterizations of C-S bond formation and cleavage enzymes in the fungus *Neurospora crassa* ergothioneine biosynthetic pathway

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Full author list of a few references:

(31) Galagan, J. E.; Calvo, S. E.; Borkovich, K. A.; Selker, E. U.; Read, N. D.; Jaffe, D.; FitzHugh, W.; Ma, L. J.; Smirnov, S.; Purcell, S.; Rehman, B.; Elkins, T.; Engels, R.; Wang, S. G.; Nielsen, C. B.; Butler, J.; Endrizzi, M.; Qui, D. Y.; Ianakiev, P.; Pedersen, D. B.; Nelson, M. A.; Werner-Washburne, M.; Selitrennikoff, C. P.; Kinsey, J. A.; Braun, E. L.; Zelter, A.; Schulte, U.; Kothe, G. O.; Jedd, G.; Mewes, W.; Staben, C.; Marcotte, E.; Greenberg, D.; Roy, A.; Foley, K.; Naylor, J.; Stabge-Thomann, N.; Barrett, R.; Gnerre, S.; Kamal, M.; Kamvysselis, M.; Mauceli, E.; Bielke, C.; Rudd, S.; Frishman, D.; Krystofova, S.; Rasmussen, C.; Metzenberg, R. L.; Perkins, D. D.; Kroken, S.; Cogoni, C.; Macino, G.; Catcheside, D.; Li, W. X.; Pratt, R. J.; Osmani, S. A.; DeSouza, C. P. C.; Glass, L.; Orbach, M. J.; Berglund, J. A.; Voelker, R.; Yarden, O.; Plamann, M.; Seiler, S.; Dunlap, J.; Radford, A.; Aramayo, R.; Natvig, D. O.; Alex, L. A.; Mannhaupt, G.; Ebbole, D. J.; Freitag, M.; Paulsen, I.; Sachs, M. S.; Lander, E. S.; Nusbaum, C.; Birren, B. *Nature* **2003**, *422*, 859.

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1. Materials and experimental procedure.

All reagents were purchased from Aldrich/Sigma if not otherwise stated. α-*N*–methyl histidine were purchased from Bachem. α–*N*,*N*–dimethyl histidine was prepared according to previously described methods.[1] Cysteine sulfinic acid standard obtained from MP Biomedicals.

A. Anaerobic Egt1 purification.

The Egt1 gene (NCU04343) was sub-cloned into the EcoRI and XhoI sites of pASK-IBA3$^+$ expression vector from IBA GmbH.

The sequence of the recombinant Egt1 is:

MGDRGPEFPSAESMTPSSALGQLKATGQHVLSKLQQQTSNADIIDIRRVAVEINLKTEITSMFRPKDGPRQ
LPTLLLYNERGLQLFERITYLEEYYLTNDEIKILTKHATEMASFIPSGAMIIELGSGNLRKVNLLLEALDNAGKAI
DYYALDLSREELERTLAQVPSYKHVKCHGLLGTYDDGRDWLKAPENINKQKCILHLGSSIDKVGITHEFILNGL
RNANEIIGETAFIEGDWRVIGEYVYDEEGGRHQAFYAPTRDTMVMGELIRSHDRIQIEQSLKYSKEESERLWSTA
GLEQVSEWTYGNEYGLHLLAKSRMSFSLIPSVYARSALPTLDDWEALWATWDVVTRQMLPQEELLEKPIKLRN
ACIFYLGHIPTFLDIQLTKTTKQAPSEPAHFCKIFERGIDPDVDNPELCHAHSEIPDEWPPVEEILTYQETVRSRLRG
LYAHGIANIPRNVGRAIWVGFEHELMHIETLLYMMLQSDKTLIPTHIPRPDFDKLARKAESERVPNQWFKIPAQE
ITIGLDDPEDGSDINKHYGWDNEKPPRRVQVAAFQAQGRPITNEEYAQYLLEKNIDKLPASWARLDNENISNGT
TNSVSGHHSNRTSKQQLPSSFLEKTAVRTVYGLVPLKHALDWPVFASYDELAGCAAYMGGRIPTFEETRSIYAY
ADALKKKKEAERQLGRTVPAVNAHLTNNGVEITPPSSPSSETPAESSSPSDSNTTLITTEDLFSDLDGANVGFHN
WHPMPITSKGNTLVGQGELGGVWEWTSSVLRKWEGFEPMELYPGYTADFFDEKHNIVLGGSWATHPRIAGRK
SFVNWYQRNYPYAWVGARVVRDLLEVDLQGDHGLSAWSHPQFEK

The amino acids colored in red are extra amino acids introduced during the sub-cloning process. The amino acid sequence colored in blue is the Strep-tag, which is used for affinity-based purification by Streptavidin resin.

The Egt1-pASK-IBA3$^+$ construct was transformed into BL21(DE3) cell and grown in 4 L LB medium (supplemented with 0.1 mM ammonium iron(II) sulfate, 100 μg/mL ampicillin). After the OD_{600} reached 0.6, anhydrotetracycline (AHT) was added to a final concentration of 200 ng/mL to induce Egt1 overexpression at 16 °C for 16 hours. Cells (7 g) were resuspended in 35 mL of anaerobic buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) in an anaerobic coy chamber. Lysozyme (1.0 mg/mL of final concentration) and DNase I (100 U/g cell) were then added into the cell suspension and the mixture was incubated on ice for 40 min with gentle agitation. The cells were disrupted by sonication (20 cycles of 30 s bursts). The supernatant and the cell debris were separated anaerobically by centrifugation at 4 °C for 10 min at 20,000 g. To the supernatant (30 mL), streptomycin sulfate was added to a final concentration of 1% (w/v %) and the mixture was mixed on ice for 30 min with gentle agitation. The white DNA precipitate was then separated by centrifugation at 20,000 g for 40 min at 4 °C. The resulting supernatant was mixed with the Streptavidin resin (10 mL) and incubated on ice for 30 min. After the cell lysate was drained by gravity, the column was washed with washing buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) until the OD_{260} was lower than 0.05. The recombinant protein was eluted with the elution buffer (2.5 mM desthiobiotin in 100 mM Tris-HCl, 150 mM NaCl buffer, pH 7.5). After the protein was concentrated anaerobically by ultrafiltration in the coy chamber, it was flash frozen by liquid nitrogen and stored at -80 °C. The typical yield is ~ 3 mg of purified Egt1 per gram of wet cells.

B. Kinetic characterization of Egt1.

Initially, we purified Egt1 as an apo-protein by including 1,10-phenanthroline in the buffer during the purification process. Apo-Egt1 does not have detectable activity based on both our oxygen consumption and ^{1}H-NMR assays. Different metals, including Fe(II), Cu(II), Mn(II), Mn(III), Co(II), Ni(II), were then used to reconstitute Egt1 and only Fe(II) reconstituted enzyme is active. In subsequent purification, apo-Egt1 was then reconstituted with Fe(II) and Fe(II) loaded Egt1 was used for activity assays discussed in this report.

Egt1 was characterized kinetically using Neofox oxygen electrode at 23 °C. A typical reaction mixture contains: 0.37 μM of Egt1, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer. When cysteine K_m was measured, 10 mM hercynine was used. When hercynine K_m was measured, 10 mM cysteine was used. The kinetic data was then analyzed by SigmaPlot.

C. Isolation and characterization of Egt1 product

An aerobic reaction assay mixtures in a 10 mL of final total volume contained 50 mM air saturated pH 8.0 KPi buffer, 1.0 mM L-hercynine, 1.5 mM L-cysteine, 1.0 mM tris(2-carboxyethyl)phosphine (TCEP), 0.2 mM ascorbate and 12 μM Egt1 protein. The mixtures were incubated at 30 °C aerobically for 2 hours. The Egt1 protein in the reaction mixture was removed by ultrafiltration. The desired product was purified by an ion-exchange column. Purified compound **4** was lyophilized and dissolved in 400 μL of D_2O. Compound **4** was analyzed using Varian 500 MHz ^{1}H-, ^{13}C-NMR spectroscopies, and high-resolution mass spectrometry. The NMR spectroscopic data for **4** are:

^{1}H-NMR (500MHz, 20 °C): δ 3.12-3.24 (m, 11H), δ 3.50 (dd, J=8.6, 13.9 Hz, 1H), δ 3.69 (dd, J=4.2, 13.9 Hz, 1H), δ 3.80 (dd, J= 3.9, 11.7, 1H), δ 3.88 (dd, J= 4.4, 8.8, 1H), δ 7.16 (s, 1 H);

^{13}C-NMR (125MHz, 20 °C): δ 25.30 (3), 50.34 (2'), 52.05 (7), 53.86 (3'), 78.12 (2), 121.49 (5), 136.01 (4), 143.06 (6), 170.60 (1), 173.08 (1'). Please refer the structure in Figure 5S for the numbering system.

High resolution ESI-MS of compound **4** (The calculated molecular weight for compound **4** as the $[M-H]^+$ form was 333.1233, and found to be 333.1234).

Cysteine sulfinic acid (a minor product from Egt1 reaction) was also purified using ion-exchange chromatography. The Egt-1 enzymatic reaction mixture was loaded onto the resin (H^+ form) and washed with H_2O. The first several H_2O washing fractions were collected and lyophilized to provide the cysteine sulfinic acid.

^{1}H-NMR (500 MHz, 20 °C, D_2O δ 4.65 ppm, pD = 7): δ 2.59 (dd, J=14.3, 8.9 Hz, 1H), δ 2.75 (dd, J=14.3, 4.0 Hz, 1H), δ 4.24 (dd, J=8.9, 4.0 Hz, 1H);

^{13}C-NMR (125 MHz, 20 °C, D_2O, pD =7): δ 47.38 (3), 55.83 (2), 170.69 (1);

High resolution ESI-MS of cysteine sulfinic acid: the calculated molecular weight as the $[M-H]^-$ form was 152.0023 and found to be 152.0033.

D. Quantify the ratio of compound 4 and 8 from Egt1 reaction.

Egt1 reaction produces two products, oxidative coupling product **4** and cysteine sulfinic acid (**8**). To quantify the ratio between these two products, 5 mL reaction was conducted and the reaction mixture contained 50 mM air saturated pH 8.0 KPi buffer, 1.0 mM L-hercynine, 1.5 mM L-cysteine, 1.0 mM tris(2-carboxyethyl)-phosphine (TCEP), 0.2 mM ascorbate, and Egt1 protein (final concentration of 10 μM). The mixture was incubated at 30 °C aerobically for 1 hour. The Egt1 protein in the reaction mixture was removed by ultrafiltration.

Based on NMR characterization of pure cysteine sulfinic acid (**8**), chemical shifts for L-cysteine sulfinic acid β hydrogen are: δ 2.54 (dd, J=9.5, 14.5 Hz, 1H), δ 2.63 (dd, J=3.8, 13.3, 0.5H) and compound **4** imidazole hydrogen chemical shift is 7.11 ppm. Direct quantification of compound **4** imidazole hydrogen and cysteine sulfinic acid β hydrogen in ^{1}H-NMR spectra would lead to an inaccurate integration because their signals are far from each other and are located at the two sides of the water signal. To avoid this issue, ethyl viologen (as shown in **Figure 9S**) was used as an internal standard to calibrate the ratio of cysteine sulfinic acid (**8**) and compound **4** because ethyl viologen has signals at both low field and high field ranges (please refer **section v** and **Figure 9S** for the compound numbering system). Ethyl viologen chemical shifts used in this analysis are: δ 1.53 (t, 7.3Hz, 6H), δ 8.38 (d, 6.4Hz, 4H), and δ 8.97(d, 6.4Hz, 4H).

Once the ratio between the coupling product **4** and ethyl viologen and the ratio between cysteine sulfinic acid **8** and ethyl viologen were determined, the ratio between coupling product **4** and cysteine sulfinic acid **8** was calculated by dividing these two ratios. Using this method, the ratio between cysteine sulfinic acid (**8**) and compound **4** was determined to be ~ 1: 12.

Both the formation of **4** and the full reduction of O_2 are four-electron processes. No other extra reductant is needed. In this reaction, the ratio between compound **4** formation and the amount of O_2 consumed is ~ 1. One of the oxygen atoms from O_2 is incorporated into **4** as the sulfoxide, it is thus reasonable to propose that the other oxygen atom ends up as H_2O. In addition, when assaying for H_2O_2 production, there is no detectable H_2O_2 formation either.

E. Determine the kinetic properties when alternative substrates were used.

To determine Egt1 substrate specificity, we have also examined Egt1 using either γ-Glu-Cys or cysteine as the substrates. The reaction was characterized using NeoFox oxygen electrode.

When hercynine and γ-Glu-Cys were the substrates, Egt1 steady-state kinetic analysis conditions were:

1. With L-hercynine concentration as the variable at 23 °C. Assay mixtures contained 2.1 μM of Egt1, 30 mM γ-Glu-Cys, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer, pH 8.0 and various amounts of L-hercynine in a total volume of 1.0 mL. The reaction was monitored by oxygen consumption using the NeoFoxy oxygen electrode. The data was fitted by SigmaPlot.
2. With γ-Glu-Cys concentration as the variable at 23 °C. Assay mixtures contained 2.1 μM Egt1, 10 mM L-hercynine, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer (pH 8.0) and various amounts of γ-Glu-Cys in a total volume of 1.0 mL. The reaction was monitored by oxygen consumption using the NeoFoxy oxygen electrode. The data was fitted by SigmaPlot.

To confirm that cysteine is the preferred substrate for Egt1, we have also conducted competitive experiment and the reaction was monitored by oxygen consumption assay. In the first 6 minutes, the reaction mixture contained 0.5 μM Egt1, 5 mM L-hercynine, 2 mM γ-Glu-Cys, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer, pH 8.0 in a total volume of 1.0 mL. There is almost no obvious oxygen concentration change. At minute 6, 2.0 mM L-cysteine was added into the above mixture and an instant drop of oxygen level supports that the Egt1 makes use of L-cysteine instead of γ-Glu-Cys as its substrate in native Egt1-catalysis.

Similarly, we have also conducted competition experiments to confirm that hercynine is the preferred substrate for Egt1. Egt1 reaction was monitored by oxygen consumption assay. In the first 6 minutes, the reaction mixture contained 0.25 μM Egt1, 10 mM L-histidine, 10 mM L-cysteine, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer (pH 8.0) in a total volume of 1.0 mL. There is almost no obvious oxygen concentration change. At minute 6, 5.0 mM L-hercynine was added into the mixture and an instant drop of oxygen level indicates the Egt1 makes use of L-hercynine instead of L-histidine as its substrate in native Egt1-catalysis.

F. Overexpression of NCU11365

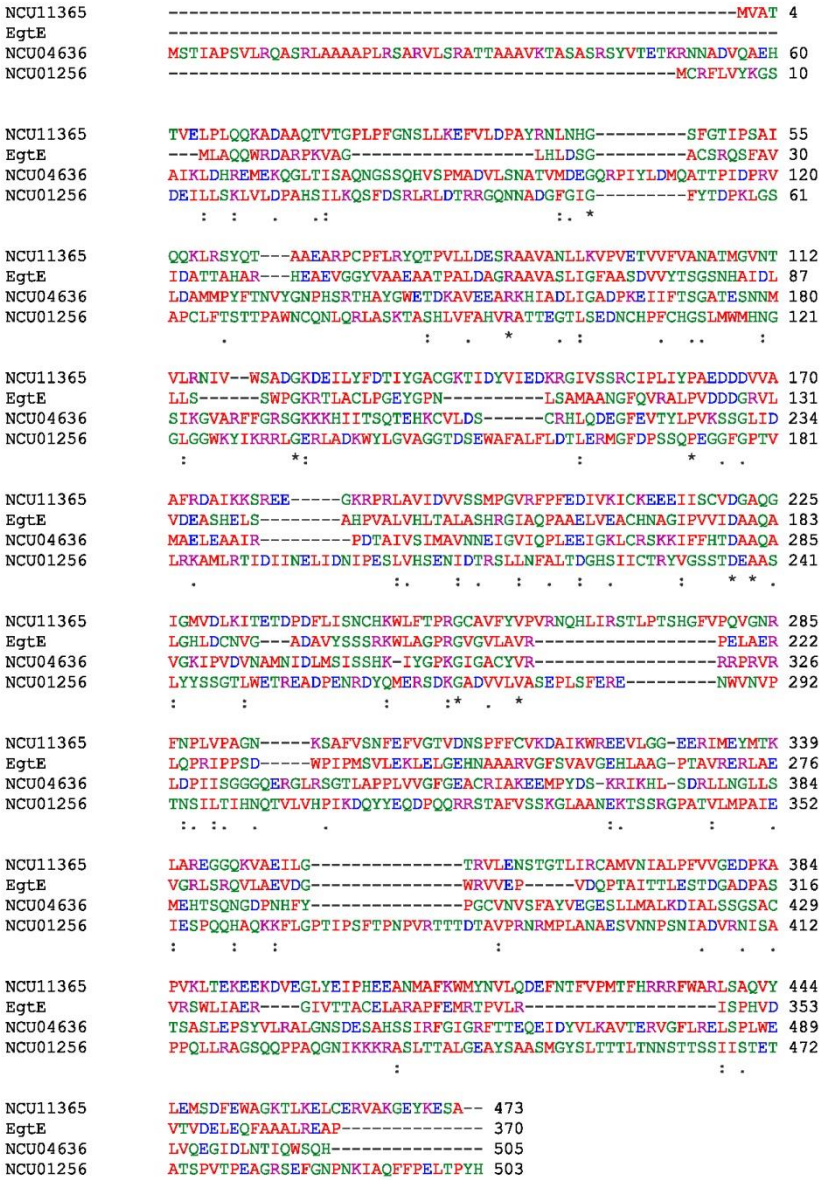
The EgtE gene (NCU11365) was sub-cloned into EcoRI and XhoI sites of the pASK-IBA3$^+$ from IBA GmbH.

The sequence of the recombinant NCU11365 is:

MGDRGPEFVATTVELPLQQKADAAQTVTGPLPFGNSLLKEFVLDPAYRNLNHGSFGTIPSAIQQKLRSYQT
AAEARPCPFLRYQTPVLLDESRAAVANLLKVPVETVVFVANATMGVNTVLRNIVWSADGKDEILYFDTIYGAC
GKTIDYVIEDKRGIVSSRCIPLIYPAEDDDVVAAFRDAIKKSREEGKRPRLAVIDVVSSMPGVRFPFEDIVKICKEE
EIISCVDGAQGIGMVDLKITETDPDFLISNCHKWLFTPRGCAVFYVPVRNQHLIRSTLPTSHGFVPQVGNRFNPLV
PAGNKSAFVSNFEFVGTVDNSPFFCVKDAIKWREEVLGGEERIMEYMTKLAREGGQKVAEILGTRVLENSTGTL
IRCAMVNIALPFVVGEDPKAPVKLTEKEEKDVEGLYEIPHEEANMAFKWMYNVLQDEFNTFVPMTFHRRRFW
ARLSAQVYLEMSDFEWAGKTLKELCERVAKGEYKESALEVDLQGDHGLSAWSHPQFEK

The amino acids colored in red are extra amino acids introduced during the sub-cloning process. The amino acid sequence colored in blue is the Strep-tag, which is used for affinity-based purification by Streptavidin resin.

The sequence alignment for NCU11365 with a few other proteins are:



The NCU11365-pASK-IBA3^{+} construct was transformed into BL21(DE3) cell and grown in 4 L LB medium (supplemented with 0.1 mM PLP and 100 μg/mL ampicillin). After the OD_{600} reached 0.6, anhydrotetracycline (AHT) was added to a final concentration of 200 ng/mL to induce NCU11365 overexpression at 16 ^{o}C for 16 hours. Cells (10 g) were resuspended in 50 mL of buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). Lysozyme (1.0 mg/mL of final concentration) and DNase I (100 U/g cell) were then added into the cell suspension and the mixture was incubated on ice for 40 min. with gentle agitation. The cells were disrupted by sonication (20 cycles of 30 s bursts). The supernatant and the cell debris were separated by centrifugation at 4 °C for 10 min at 20,000 g. To the supernatant (50 mL), streptomycin sulfate was added to a final concentration of 1% (w/v %) and the mixture was mixed on ice for 30 min with gentle

agitation. The white DNA precipitate was then separated by centrifugation at 20,000 g for 30 min at 4 ℃. The resulting supernatant was mixed with the Streptavidin resin (10 mL) and incubated on ice for 30 min. After the cell lysate was drained by gravity, the column was washed with washing buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) until the OD_{260} is lower than 0.05. The recombinant protein was eluted with the elution buffer (2.5 mM desthiobiotin in 100 mM Tris-HCl buffer, pH 7.5). After the protein was concentrated by ultrafiltration, it was flash frozen by liquid nitrogen and stored at -80 °C. The typical yield is ~ 5 mg of purified NCU11365 per gram of wet cells.

G. Isolation and characterization of ergothioneine (5) from NCU11365 reaction.

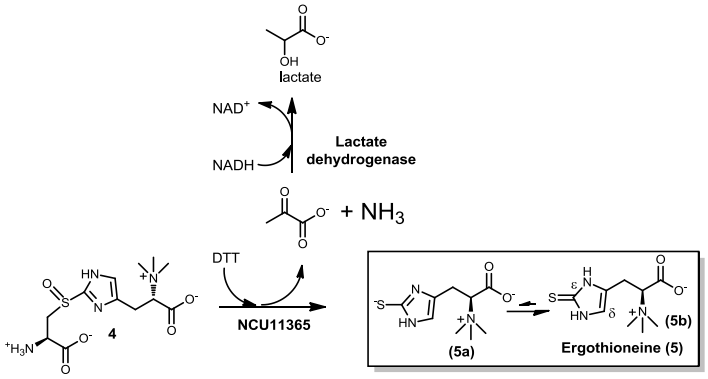
A reaction assay mixtures in a 10 mL of final total volume contained 50 mM pH 8.0 KPi buffer, 2 mM compound **4**, 2 mM DTT and 1 μM NCU11365 protein. The mixtures were incubated at 28 °C for 1 hour. The NCU11365 protein in the reaction mixture was removed by ultrafiltration. The desired product was purified by HPLC (C18 reversed phase semi−prep column, waters, 250 × 10 mm, mobile phase: 1 ml/min flow of H_2O containing 2% acetonitrile). Purified ergothioneine (**5)** was lyophilized and dissolved in 400 μL of D_2O. Ergothioneine (**5)** was analyzed using Varian 500 MHz ^{1}H-NMR spectroscopies, and high-resolution mass spectrometry.

^{1}H-NMR (500MHz, 20 °C) of ergothioneine (**5)**: δ 2.99-3.16 (m, 11H), δ 3.73 (dd, J=3.9, 11.7 Hz, 1H), δ 6.64 (s, 1 H);

^{13}C-NMR (125MHz, 20 °C) of ergothioneine (**5**): δ 22.73 (3), 52.04 (7), 76.99 (2), 115.22 (5), 123.78 (4), 155.80 (6), 169.98(1);

High resolution ESI-MS of ergothioneine (**5**): Calculated molecular weight for compound **5** as $[M-2H]^-$ (negative mode) form was 228.0812, and found 228.0820.

H. NCU11365 kinetic characterization by Lactate dehydrogenase coupled assay.



Because pyruvate is produced as one of the side-products along with ergothioneine in NCU11365 reaction, the NCU11365 kinetics was measured by coupling it with lactate dehydrogenase and monitoring the consumption of NADH consumption rate. A typical assay mixture contained 12.5 nM of NCU11365, 0.13 mM NADH, 1.0 mM DTT, 22.5 U/mL LDH (2000× compared to NCU11365 activity in the assay) in 50 mM KPi buffer, pH 8.0 and various amounts of NCU11365 substrate in a total volume of 1.0 mL. The reaction was monitored at 340 nm using the Varian Cary 100 Bio UV-vis spectrometer. The data was fitted by SigmaPlot.

2. Supporting information (protein purification, kinetic characterization, product isolation and characterization by NMR and mass spectrometry):

i. The UV-visible spectrum and SDS-PAGE gel of the anaerobically purified Egt1.

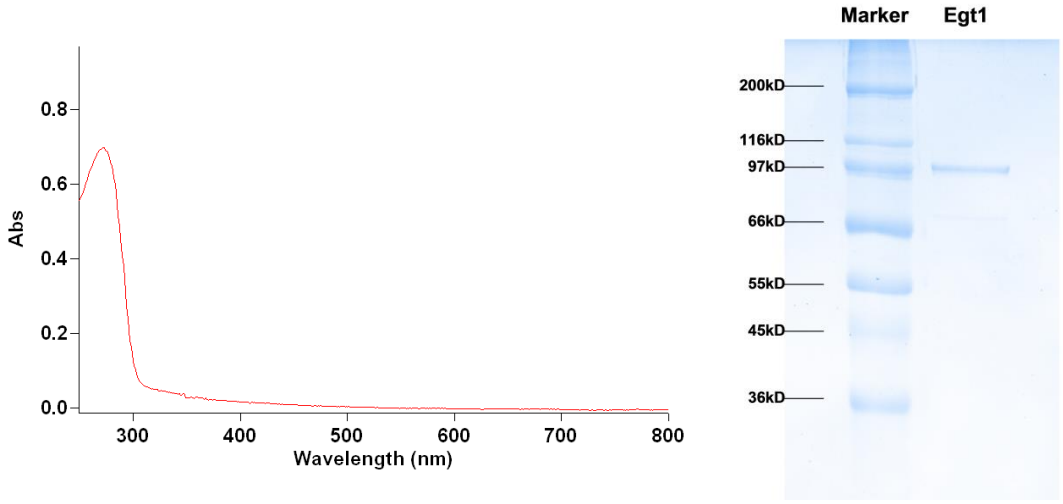


Figure 1S. 1) The UV-visible spectrum of the anaerobically purified Egt1 using Streptavidin resin from IBA, Inc. 2) SDS-PAGE gel of the purified Egt1 protein.

ii. Egt1 steady-state kinetic analysis using L-hercynine and L-cysteine as the substrates.

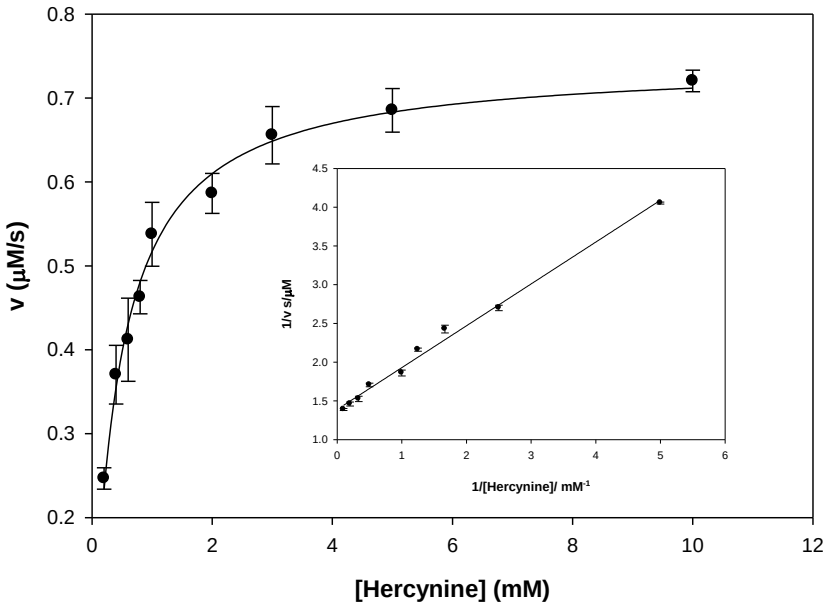


Figure 2S-1. Egt1 steady-state kinetic analysis using L-hercynine concentration as the variable at 23 °C. Assay mixtures contained 0.37 μM of Egt1, 10 mM L-cysteine, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer, pH 8.0 and various amounts of L-hercynine in a total volume of 1.0 mL. The reaction was monitored using oxygen consumption assay by the NeoFox oxygen electrode. The data was fitted by SigmaPlot.

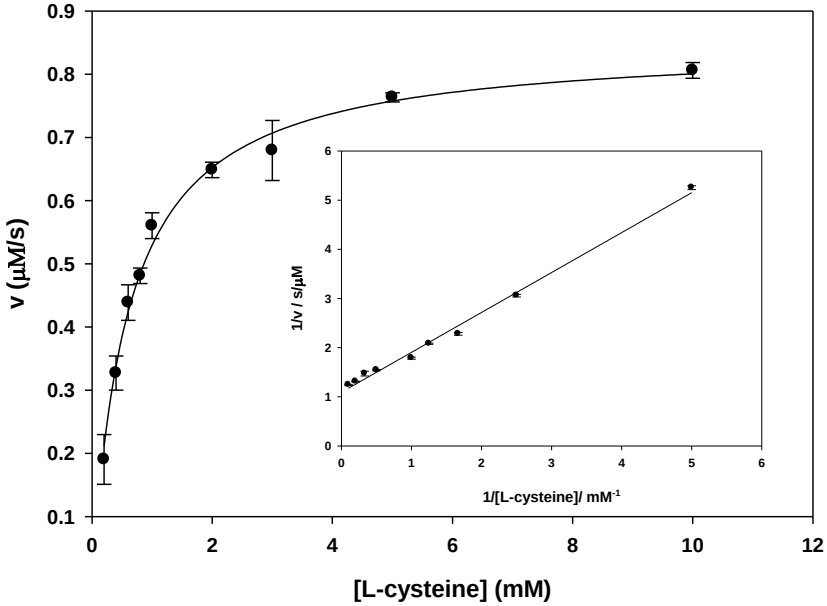


Figure 2S-2. Egt1 steady-state kinetic analysis using L-cysteine concentration as the variable at 23 °C. Assay mixtures contained 0.37 μM of Egt1, 10 mM L-hercynine, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer, pH 8.0 and various amounts of L-cysteine in a total volume of 1.0 mL. The reaction was monitored by oxygen consumption using the NeoFox oxygen electrode. The data was fitted by SigmaPlot.

iii. Characterizations of Egt1 when L-hercynine and L-cysteine were the substrates (^{1}H-NMR, ^{13}C-NMR, high-resolution mass spectrometry).

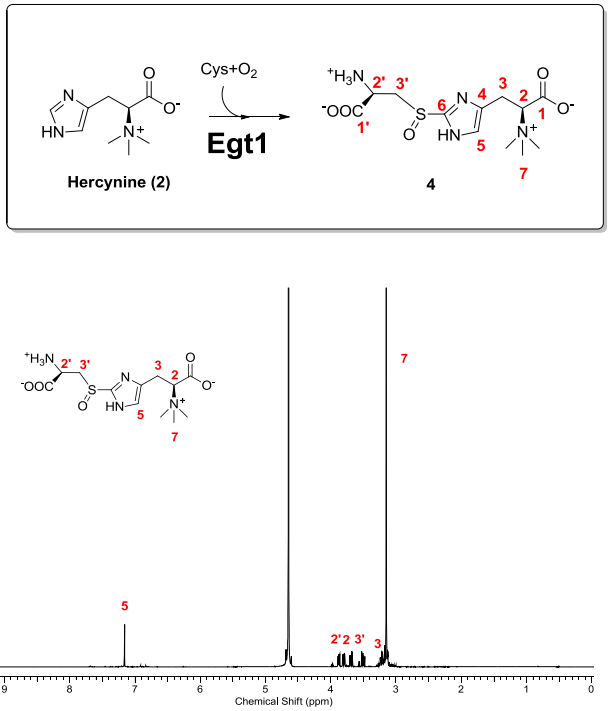


Figure 3S. ^{1}H-NMR of the purified compound **4**.

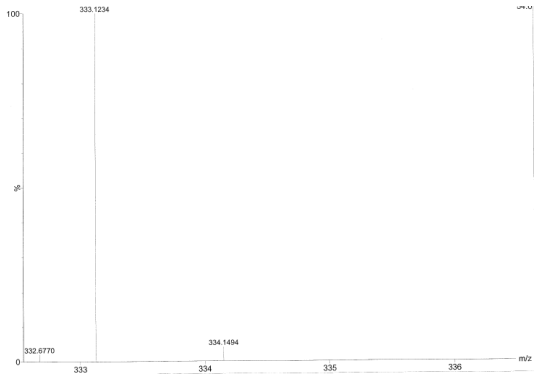


Figure 4S. The high resolution ESI-MS of compound **4** (The calculated molecular weight for compound **4** as the $[M-H]^+$ form was 333.1233, and found to be 333.1234).

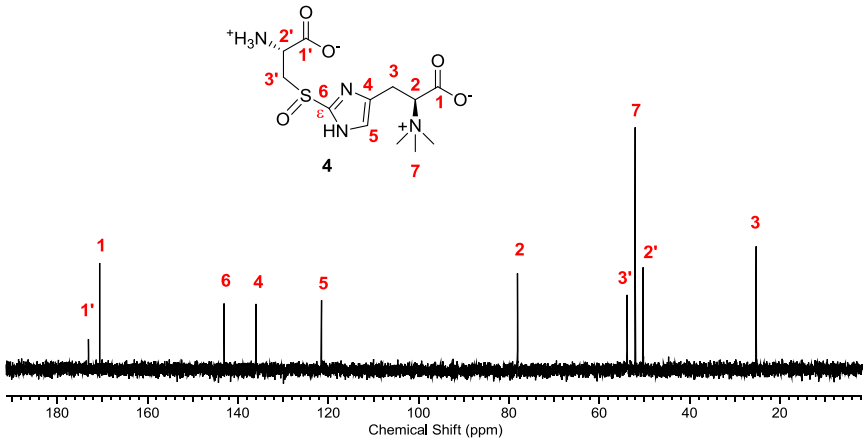


Figure 5S. ^{13}C-NMR of compound **4**.

iv. Characterization of the minor product, cysteine sulfinic acid in Egt1 reaction.

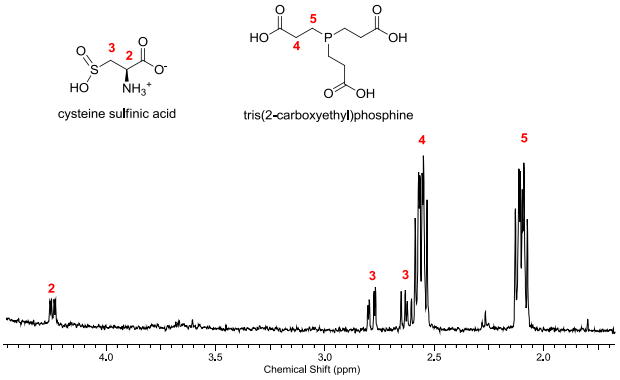


Figure 6S. ^{1}H-NMR spectrum of the partially purified cysteine sulfinic acid (**8**) from the Egt1 reaction mixture (using L-hercynine and L-cysteine as substrates). Besides the signals corresponding to the cysteine sulfinic acid (**8**), the additional NMR signals are from TCEP.

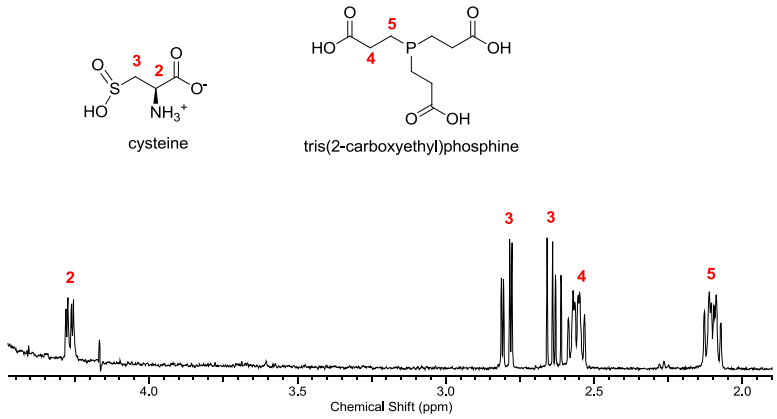


Figure 7S. When the isolated cysteine sulfinic acid (**8**) was spiked with the authentic cysteine sulfinic acid standard obtained from MP Biomedicals., the signals labeled as **2** and **3** in the ^{1}H-NMR spectrum increased compared to those in Figure 6S.

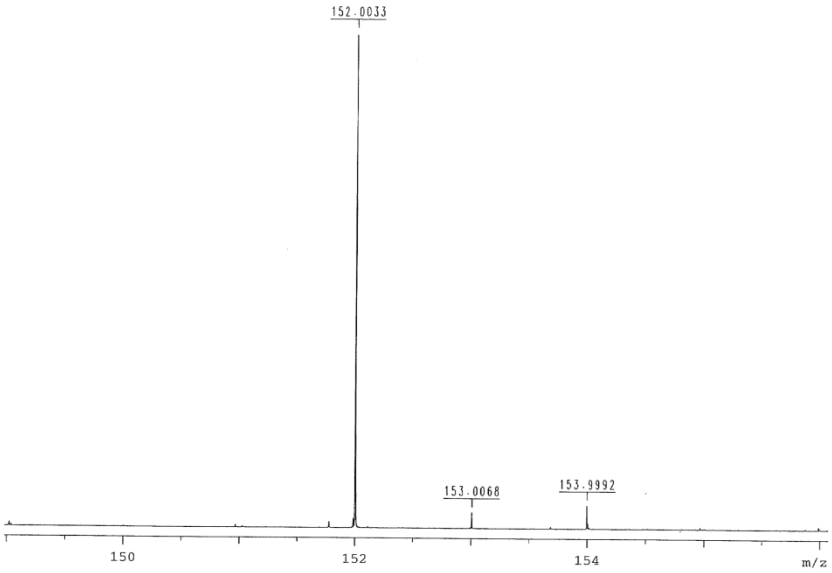
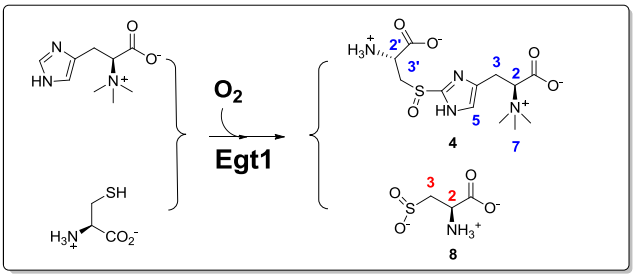


Figure 8S. The high resolution ESI-MS of the partially purified cysteine sulfinic acid (**8**) (the calculated molecular weight for compound **8** as $[M-H]^-$ form was 152.0023 and found to be 152.0033).

v. Quantify the ratio of cysteine sulfinic acid (8) and compound 4 in Egt1 reaction when L-cysteine and L-hercynine were used as substrates



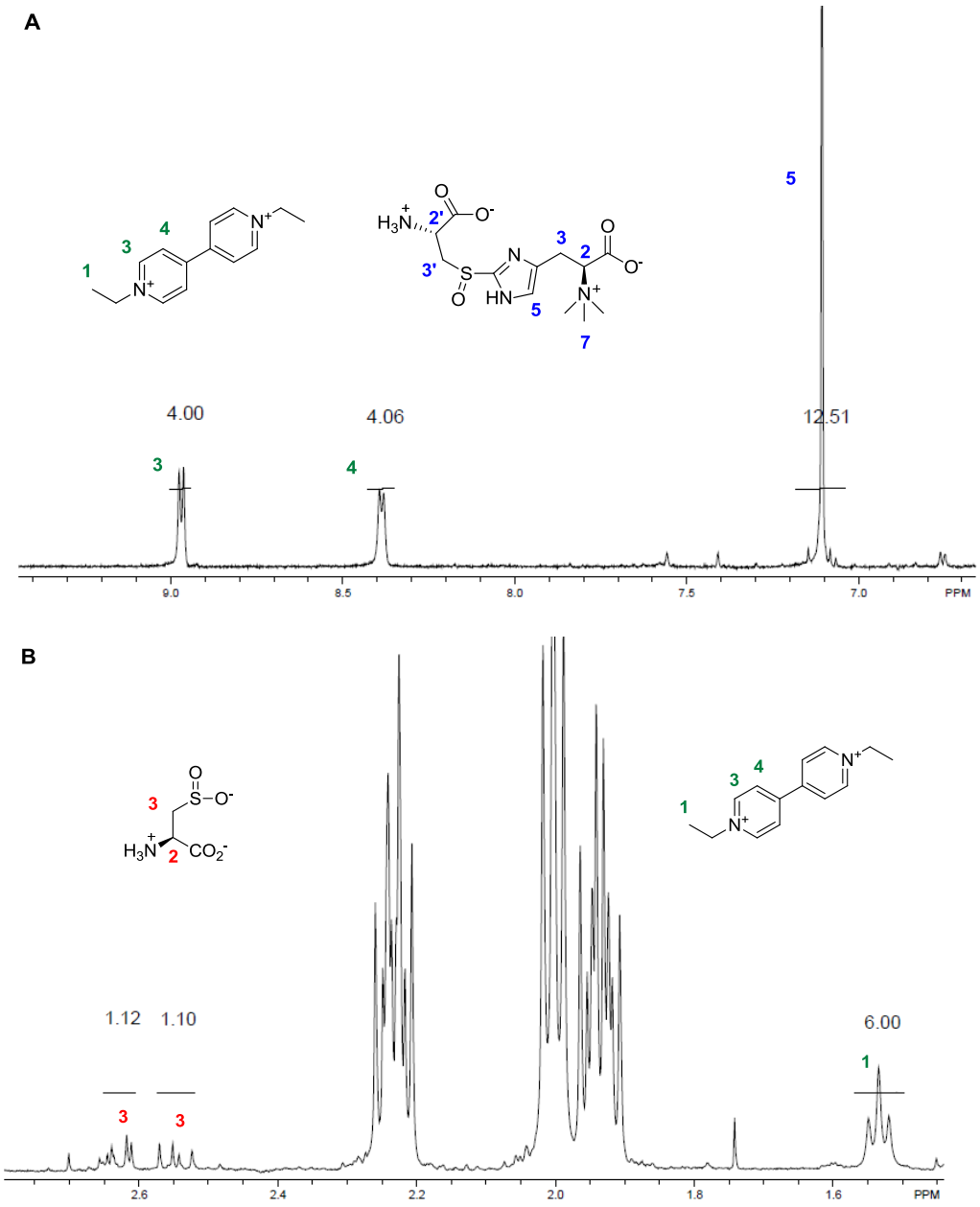


Figure 9S. ^{1}H-NMR spectrum of the Egt1-reaction using L-hercynine and L-cysteine as substrates. **A:** Expanded regions between 6.7 ppm - 9.5 ppm. **B:** Expanded regions between 1.4 - 2.8 ppm regions. To quantitatively measure the ratio between compound **4** and cysteine sulfinic acid (**8**), ethyl viologen was added as an internal standard to improve the accuracy of signal quantification. Ethyl viologen has two signals in the 8 – 9.5 ppm region (labeled as 3 and 4, colored green in the spectrum) and signals at ~ 1.5 ppm region (labeled as 1 and colored green in the spectrum). The ratio between compound **4** and the ethyl viologen signals was calculated by measuring the ratio between compound **4** H-5 signal (labeled as **5** and colored blue in the spectrum) and the ethyl viologen aromatic hydrogen signals. The ratio between cysteine sulfinic acid and the ethyl viologen signals was calculated by measuring the ratio between cysteine sulfinic acid β-hydrogen (labeled as 3 and colored red in the spectrum) and the ethyl viologen ethyl group hydrogen signals (labeled as 1 and colored green in the spectrum). Based on the integration using ethyl viologen as an internal standard, the ratio of cysteine sulfinic acid (**8**) and compound **4** was ~ 1: 12.

vi. Egt1 steady-state kinetic analysis using L-hercynine and γ-Glu-Cys as the substrates.

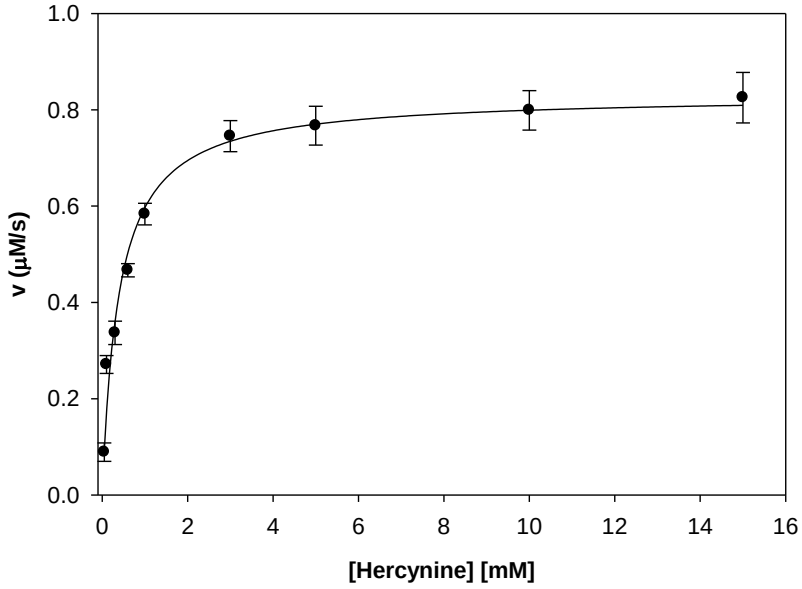


Figure 10S-1. Egt1 steady-state kinetic analysis with L-hercynine concentration as the variable at 23 °C. Assay mixtures contained 2.1 μM of Egt1, 30 mM γ-Glu-Cys, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer, pH 8.0 and various amounts of L-hercynine in a total volume of 1.0 mL. The reaction was monitored by oxygen consumption using the NeoFox oxygen electrode. The data was fitted by SigmaPlot.

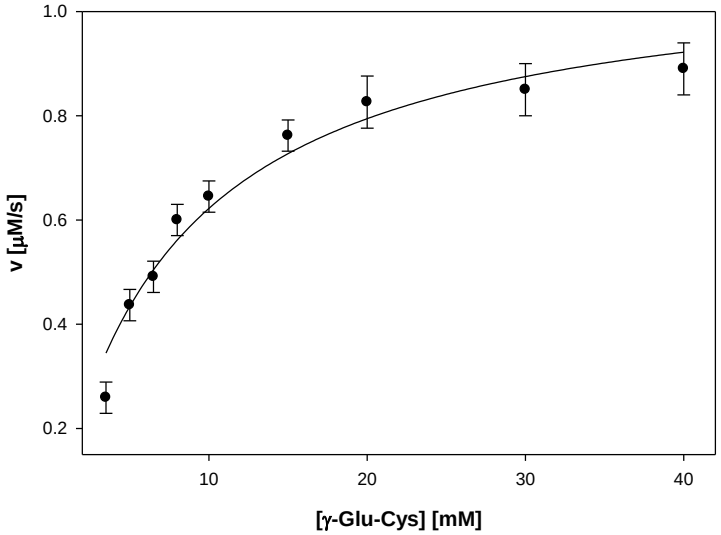


Figure 10S-2. Egt1 steady-state kinetic analysis with γ-Glu-Cys concentration as the variable at 23 °C. Assay mixtures contained 2.1 μM Egt1, 10 mM L-hercynine, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer (pH 8.0) and various amounts of γ-Glu-Cys in a total volume of 1.0 mL. The reaction was monitored by oxygen consumption using the NeoFox oxygen electrode. The data was fitted by SigmaPlot. Clearly, γ-Glu-Cys is not a good substrate for Egt1.

vii. Competition between L-cysteine and γ-Glu-Cys for Egt1 reaction in the presence of L-hercynine.

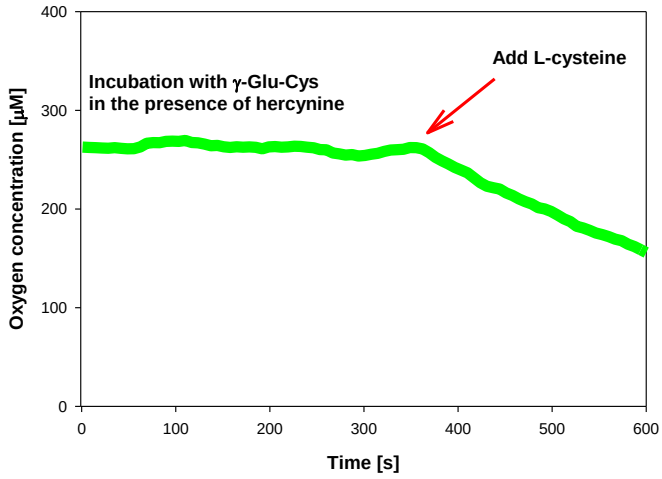


Figure 11S. Egt1 reaction monitored by oxygen consumption assay. In the first 6 minutes, the reaction mixture contained 0.5 μM Egt1, 5 mM L-hercynine, 2 mM γ-Glu-Cys, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer, pH 8.0 in a total volume of 1.0 mL. There is almost no obvious oxygen concentration change. At minute 6, 2.0 mM L-cysteine was added into the above mixture and an instant drop of oxygen level indicates the Egt1 makes use of L-cysteine instead of γ-Glu-Cys as its substrate in native Egt1-catalysis.

viii. Competition between L-hercynine and L-histidine for Egt1 reaction in the presence of L-cysteine.

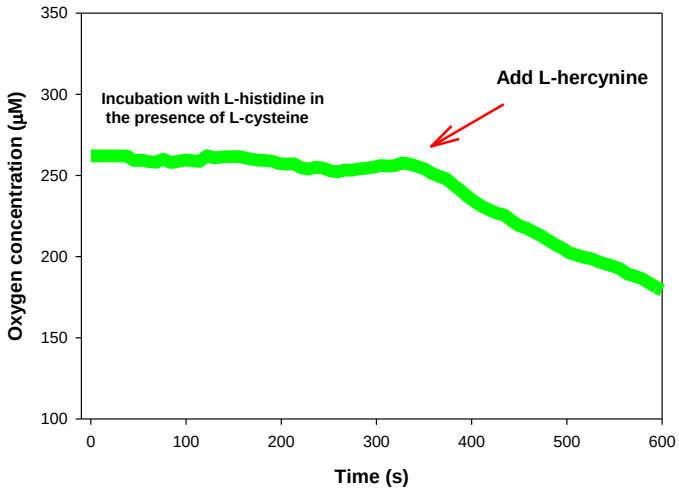


Figure 12S. Egt1 reaction monitored by oxygen consumption assay. In the first 6 minutes, the reaction mixture contained 0.25 μM Egt1, 10 mM L-histidine, 10 mM L-cysteine, 1.0 mM TCEP, 0.2 mM ascorbate in 50 mM air-saturated HEPES buffer (pH 8.0) in a total volume of 1.0 mL. There is almost no obvious oxygen concentration change. At minute 6, 5.0 mM L-hercynine was added into the mixture and an instant drop of oxygen level indicates the Egt1 makes use of L-hercynine instead of L-histidine as its substrate in native Egt1-catalysis.

ix. The UV-visible spectrum and SDS-PAGE gel of purified NCU11365.

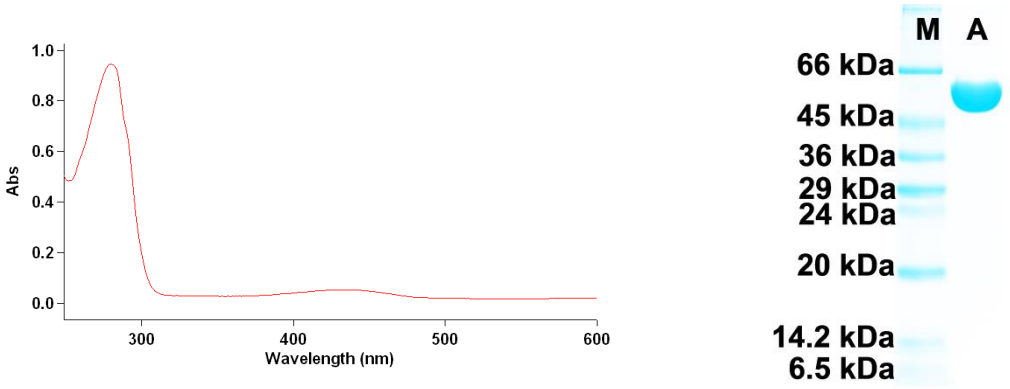


Figure 13S. Left: The UV-visible spectrum of the anaerobically purified NCU11365 using Streptavidin resin from IBA, Inc. The purified NCU11365 contains a typical PLP peak at 420 nm. Right: SDS-PAGE gel of the aerobically purified NCU11365. Lane M, Protein Ladder; Lane A, Streptavidin resin purified strep-tagged NCU11365 protein.

x. NMR and HRMS characterization of ergothioneine (5) from NCU11365 reaction.

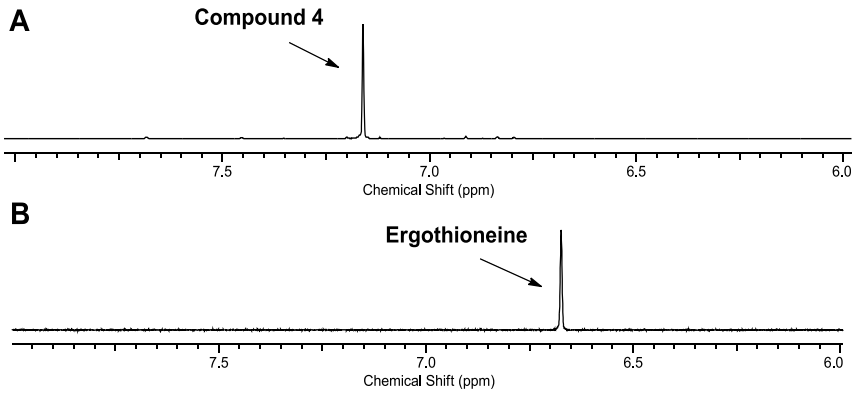


Figure 14S. 1H-NMR assay of EgtE reaction (chemical shifts of imidazole ring H-atoms). Compounds **4** (**A**) and ergothioneine (**5**) formed from NCU11365 reaction (**B**) have different chemical shift for their imidazole ring hydrogen atoms.

NMR spectroscopic data for ergothioneine (**5)**:

^{1}H-NMR (500MHz, 20 °C) of ergothioneine (**5)**: δ 2.99-3.16 (m, 11H), δ 3.73 (dd, J=3.9, 11.7 Hz, 1H), δ 6.64 (s, 1 H);

^{13}C-NMR (125MHz, 20 °C) of ergothioneine (**5)**: δ 22.73 (3), 52.04 (7), 76.99 (2), 115.22 (5), 123.78 (4), 155.80 (6), 169.98(1);

High resolution ESI-MS of ergothioneine: Calculated molecular weight for compound **5** as $[M-2H]^-$ (negative mode) form was 228.0812, and found 228.0820.

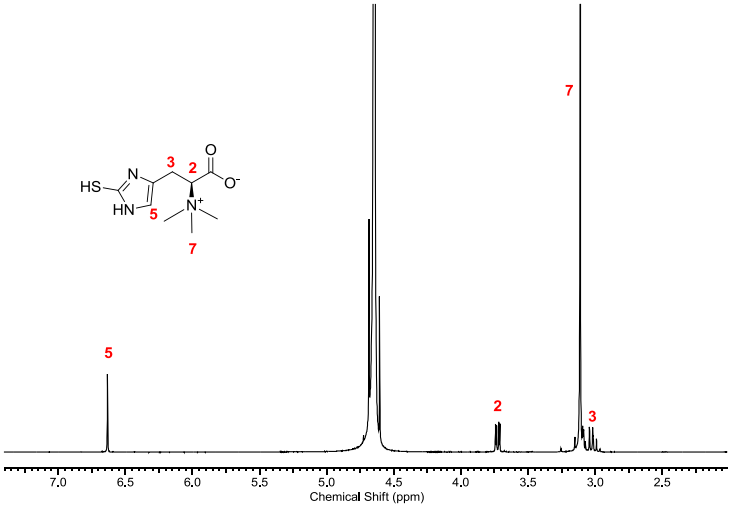


Figure 14S-A. ^{1}H-NMR spectrum of purified ergothioneine (**5**).

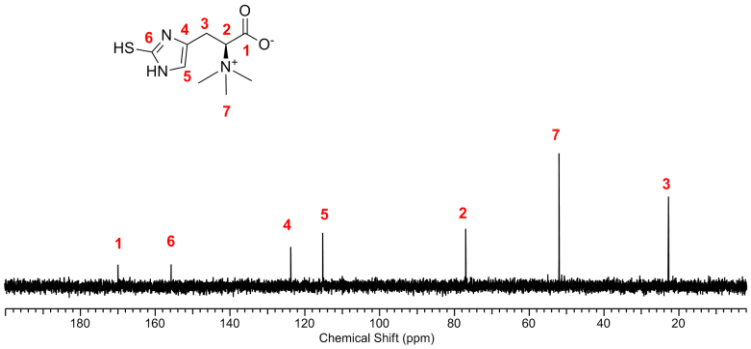


Figure 14S-B. ^{13}C-NMR spectrum of purified ergothioneine (**5**).

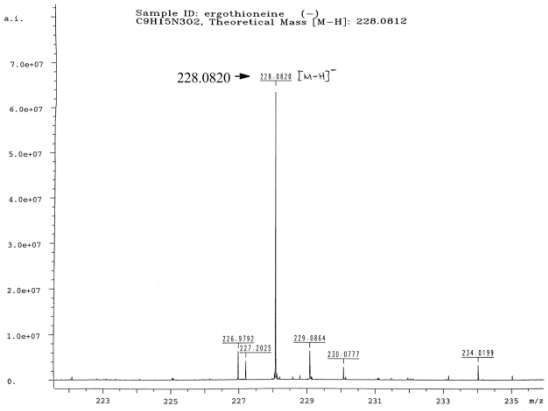


Figure 14S-C. HRMS characterization of purified ergothioneine (**5**, calculated molecular weight for compound **5** as [M-2H]$^{-}$ form (negative mode) was 228.0812, and found 228.0820).

xi. Lactate dehydrogenase coupled assay for NCU11365 kinetic determination.

The kinetics parameters obtained for NCU11365 from the lactate dehydrogenase assay is: K_M=194.7±9.8 μM for compound **4** and k_{cat} =684.4 ± 12.2/min.

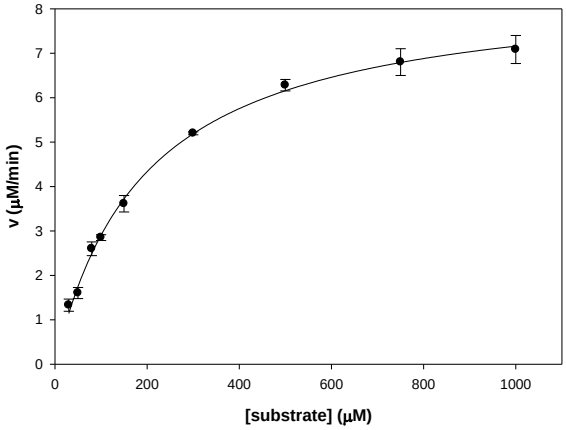


Figure 15S. NCU11365 steady-state kinetic analysis with varying substrate concentrations at 25 °C. Assay mixtures contained 12.5 nM of NCU11365, 0.13 mM NADH, 1.0 mM DTT, 22.5 U/mL LDH (1000X compared to NCU11365 activity in the assay) in 50 mM Kpi buffer, pH 8.0 and various amounts of NCU11365 substrate in a total volume of 1.0 mL. The reaction was monitored at 340 nm using the Varian Cary 100 Bio UV-vis spectrometer. The data was fitted by SigmaPlot.