Bioinformatic and Biochemical Characterizations of C–S Bond Formation and Cleavage Enzymes in the Fungus *Neurospora crassa* Ergothioneine Biosynthetic Pathway

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**Supporting Information**

**ABSTRACT:** Ergothioneine is a histidine thiol derivative. Its mycobacterial biosynthetic pathway has five steps (EgtA-EgtE catalysis) with two novel reactions: a mononuclear nonheme iron enzyme (EgtB) catalyzed oxidative C–S bond formation and a PLP-mediated C–S lyase (EgtE) reaction. Our bioinformatic and biochemical analyses indicate that the fungus *Neurospora crassa* has a more concise ergothioneine biosynthetic pathway because its nonheme iron enzyme, Egt1, makes use of cysteine instead of γ-Glu-Cys as the substrate. Such a change of substrate preference eliminates the competition between ergothioneine and glutathione biosyntheses. In addition, we have identified the *N. crassa* C–S lyase (NCU11365) and reconstituted its activity in vitro, which makes the future ergothioneine production through metabolic engineering feasible.

Sulfur-containing molecules are widely distributed in nature, including amino acids, enzyme cofactors, antioxidants, nucleotides, and numerous secondary metabolites. Their biosyntheses make use of many novel transformations. Ergothioneine is a thiol–imidazole-containing amino acid, and its first biosynthetic pathway in *Mycobacterium smegmatis* was reported in 2010 (EgtA–EgtE catalysis in Scheme 1A). The first step is the methylation of histidine to hercynine (2) catalyzed by EgtD. EgtA is γ-glutamylcysteine synthase catalyzing the condensation between glutamate and cysteine to γ-glutamlycysteine (γ-Glu-Cys). EgtB then catalyzes the oxidative coupling between hercynine and γ-Glu-Cys to form 3. An amidotransamidase (EgtC) then hydrolyzes 3 to form S-(β-amino-β-carboxethyl)ergothioneine (4). The last enzyme in ergothioneine biosynthesis is a putative PLP-dependent C–S lyase (EgtE) whose activity remains to be verified in vitro. Humans obtain ergothioneine from our diet and enrich it to as high as millimolar concentrations in many parts of our body using an ergothioneine-specific transporter. Ergothioneine’s reduction potential ($E'_0 = -0.06 \text{ V}$) is significantly higher than glutathione ($E'_0 = -0.25 \text{ V}$) and plays many beneficial roles in human health. One of its roles is to scaveng e reactive oxidative species. Its was also suggested to be involved in heavy metal detoxification.

Due to ergothioneine’s beneficial roles to human health, there is a long-standing interest in developing efficient ergothioneine production methods. The discovery of the *M. smegmatis* ergothioneine biosynthetic pathway (EgtA–EgtE catalysis, Scheme 1A) in 2010 provides an opportunity for ergothioneine production through metabolic engineering. To realize this potential, two barriers need to be addressed. First, one of the EgtB substrates is γ-Glu-Cys, which is part of the glutathione biosynthesis (Scheme 1A). As one of the most important redox buffers inside the cell, glutathione has an intracellular concentration up to 10 mM, and it is essential to cellular survival. As a result, such competition is not desirable for ergothioneine production. Second, previous EgtE protein overexpression attempts were unsuccessful. In this work, the above two issues were resolved.

Ovothiol (7, Scheme 1B) is another thiol–imidazole-containing amino acid. The enzyme responsible for the oxidative C–S bond formation in ovothiol biosynthesis (OvoA, Scheme 1B) is also a mononuclear nonheme iron enzyme. Recently, we demonstrated that when OvoA’s native substrate histidine is replaced by hercynine, OvoA can catalyze a one-step $2 \rightarrow 4$ transformation (Scheme 1B). Subsequent detailed biochemical characterization of OvoA enzyme revealed that when histidine is replaced by hercynine, $k_{\text{cat}}/K_m$ changes by 2-fold (from $572 \pm 20 \text{ min}^{-1}$ to $270 \pm 5 \text{ min}^{-1}$), while $K_m$ for cysteine increases from...
OvoA and EgtB are related in sequence, while they are clearly segregated into different clusters. Among the fungal genes, one of the genes (Egt1 from N. crassa) was proposed to be involved in fungal ergothioneine biosynthesis because its knockout in N. crassa makes the mutant much more sensitive to oxidative stress. Thus far, none of the proposed fungal ergothioneine biosynthetic enzymes has been biochemically characterized in vitro.

To examine whether fungal EgtB homologues are indeed biochemically distinct from both EgtB and OvoA as suggested by the sequence similarity network analysis (Figure 1), we subcloned the fungus N. crassa Egt1 gene to pASK-IBA3 vector and overexpressed it in E. coli. The purified protein does not have clear UV–vis spectroscopic features, which is consistent with the properties of nonheme iron enzymes (Figure 1S, Supporting Information). The amount of iron was quantified using iron titration method and Fe²⁺ reconstituted Egt1 has ~0.90 ± 0.05 Fe per Egt1 monomer. Using hercynine and cysteine as substrates, Egt1 was analyzed by three different assays (Figure 2). NeoFox oxygen electrode was used to directly measure the oxygen consumption rate. In air-saturated HEPES buffer (~250 μM of oxygen), the Egt1 kinetic parameters are $k_{cat}$ of 136 ± 4 min⁻¹ and $K_m$ of 436 ± 30 μM for hercynine, $K_m$ of 603 ± 40 μM for Cys (Figure 2S, Supporting Information). More importantly, the $^3$H NMR assay suggested that the oxidative coupling between cysteine and hercynine occurs at the imidazole ε-position (Egt1 reaction in Scheme 1A and Figure 2A). The signals with chemical shifts of 6.84 and 7.62 ppm are from the hercynine imidazole H atoms. When hercynine and cysteine were used as the Egt1 substrates, the coupling product has a chemical shift at 7.12 ppm (Figure 2A), which is consistent with a one-step 2 → 4 transformation (Egt1 catalysis in Scheme 1A, Figure 2A, Figures 3S–5S, Supporting Information).

In oxygenase- and oxidase-catalyzed reactions, it is common to have uncoupling between the oxygen consumption and the production of the desired product. When hercynine and cysteine were used as the substrates, the formation of cysteine sulfenic acid (8) is the major OvoA reaction and accounts for ~60% of oxygen consumption (Scheme 1B). When compound 4 formation rate
and oxygen consumption rate in Egt1-catalysis were analyzed quantitatively, under the assay conditions used, the oxygen consumption rate ($84 \pm 2 \text{ min}^{-1}$) is slightly faster than the oxidative product 4 formation rate ($77 \pm 2 \text{ min}^{-1}$, Figure 2B), which suggests that in Egt1-catalysis, the 2 $\rightarrow$ 4 transformation is the dominant reaction (~92% of the oxygen consumed). To examine whether the remaining ~8% of the oxygen consumption is due to cysteine oxidation reactions, Egt1-catalysis was further characterized using $^{13}$C NMR and [β-$^{13}$C]-Cys were used to replace Cys. Indeed, besides the oxidative coupling product 4 (54.5 ppm, Figure 2C), cysteine sulfinic acid (8, 57.9 ppm, Figure 2C) was the other product (Figures 6S–8S, Supporting Information). The ratio between 4 and 8 is ~12:1 (Figure 9S, Supporting Information).

To provide further evidence supporting the 2 $\rightarrow$ 4 transformation as the native Egt1-chemistry, Egt1 substrate specificities were examined using hercynine, histidine, cysteine, or γ-Glu-Cys as the substrates and in various combinations. In air-saturated HEPES buffer (~250 μM of oxygen), the reaction kinetics was measured by NeoFox oxygen electrode. The kinetic parameters for the various combinations are as follows: (A) when cysteine and hercynine are the substrates, $k_{\text{obs}}$ of 136 ± 4 min$^{-1}$ and a $K_m$ of 436 ± 30 μM for hercynine, a $K_m$ of 603 ± 40 μM for Cys; (B) when histidine and cysteine are the substrates, $k_{\text{obs}}$ is at least 100-fold less than the case using the Cys and hercynine combination (for these reasons, the detailed kinetic parameters were not measured for this case); (C) when γ-Glu-Cys and hercynine are the substrates, $k_{\text{obs}}$ of 27.7 ± 1.6 min$^{-1}$ and $K_m$ of 7.68 ± 1.11 mM for γ-Glu-Cys, $K_m$ of 390 ± 58 μM for hercynine; (D) when γ-Glu-Cys and histidine are the substrates, similar to the case of histidine and cysteine combination, the rate is close to background level and the kinetic parameters were not measured (Figure 10S, Supporting Information). These comparative kinetic studies highly suggest that the native substrates for Egt1 enzyme are hercynine and cysteine (Egt1 catalysis in Scheme 1A). When hercynine is replaced by histidine, the reaction rate reduces by at least 2 orders of magnitude. In addition, Egt1 has ~62-fold greater specificity ($k_{\text{obs}}/K_m$) for i-cysteine relative to γ-Glu-Cys.

In ergothioneine biosynthesis, there are two novel reactions: the oxidative C–S bond formation and the subsequent C–S bond cleavage reaction, which result in a net transfer of the sulfur atom from cysteine to histidine imidazole side-chain (Scheme 1 and Supporting Information). In the M. smegmatis ergothioneine biosynthetic pathway, in vitro activities of EgtA, EgtB, EgtC, and EgtD enzymes have been established. The proposed C–S lyase activity (EgtE) remains to be verified because EgtE has not yet been successfully expressed in E. coli. To identify the N. crassa EgtE homologue, we searched for genes that are predicted to interact with Egt1 using the String database based on gene co-occurrence, fusion, and gene neighborhood information (http://string-db.org/), and the NCU01256 gene was identified, and it was predicted to be a PLP-dependent enzyme. We subcloned it into pASK-IBA3+ vector and overexpressed it in E. coli BL21(DE3) strain; the purified NCU01256 protein does not have the desired C–S lyase activity. This result implies that a homologue of NCU01256 in N. crassa might be the desired C–S lyase. We then searched the N. crassa genome (http://mips.helmholtz-muenchen.de/genome/proj/ncrassa/) using NCU01256 as the query sequence, and two potential PLP-containing proteins (NCU04636 and NCU11365, Supporting Information) were identified. Similarly, we have cloned them into a pASK-IBA3+ vector, and the proteins were overexpressed in E. coli BL21(DE3) strain. Subsequent biochemical characterization revealed that NCU11365 is the C–S lyase (Figures 13S–15S, Supporting Information). The UV–vis spectrum of the isolated NCU11365 is consistent with the presence of a PLP cofactor (Figure 13S, Supporting Information). NCU11365 protein was characterized by two different assays: $^1$H NMR assay and a colorimetric assay. In the $^1$H NMR spectrum, the imidazole hydrogen atoms are indicative of the Cβ bond cleavage reaction, which results in a net transfer of the sulfur atom form cysteine to histidine imidazole side-chain. Significant changes were observed in the chemical shifts of the histidine imidazole side-chain, indicating a Cβ bond cleavage reaction and the formation of ergothioneine thiol-imidazole. The reaction rate can then be monitored by examining the imidazole hydrogen atoms.
mononuclear nonheme iron enzymes in ergothioneine and ovothiol biosyntheses (EgtB, OvoA, and Egt1) are related in sequence. However, more detailed investigation using sequence-similarity network analysis indicated that they are at different subgroups in sequence space at an E value cutoff of 1 × 10−50. Biochemical analysis results are fully compatible with the bioinformatic predictions. The differences among them are their substrate preferences and product C=S bond regioselectivity. EgtB of the M. smegmatis pathway (Scheme 1A) uses herycynine and γ-Glu-Cys as its substrates, and it is extremely specific in terms of substrate specificity.26 OvoA in ovothiol biosynthesis has a relaxed substrate specificity (Scheme 1B).25,26 Interestingly, Egt1 from fungus N. crassa prefers herycynine and cysteine and catalyzes a one-step 2 → 4 transformation (Scheme 1A). Such discovery is of practical importance because the ergothioneine and glutathione biosynthetic pathways are uncoupled and they do not compete with each other anymore in N. crassa. Thus, the N. crassa ergothioneine biosynthetic pathway is a more stable platform than the mycobacterial one for ergothioneine production through metabolic engineering. In addition, the C=S lyase (NCU11365) was successfully identified through genome mining, and the whole ergothioneine biosynthetic pathway was now reconstituted in vitro. Besides genome mining, and the whole ergothioneine addition, the C=S bond regioselectivity for ergothioneine production through metabolic engineering. In addition, the C=S lyase (NCU11365) was successfully identified through genome mining, and the whole ergothioneine biosynthetic pathway was now reconstituted in vitro. Besides its potential application in ergothioneine production through metabolic engineering, the mononuclear nonheme iron enzymes catalyzing oxidative C=S bond formations (EgtB, OvoA, and Egt1) are distinct from currently known biological C=S bond formation reactions.17,25,34–38 Future studies will focus on detailed mechanistic studies of these novel transformations.

■ ASSOCIATED CONTENT

4 Supporting Information

Experimental procedure, enzyme characterization data, 1H and 13C NMR of compounds 4 and 5, and kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): A patent related to this work has been submitted.

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