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Regio-selectivity of the Oxidative C-S Bond Formation in Ergothioneine and Ovothiol Biosyntheses

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Abstract

Ergothioneine (5) and ovothiol (8) are two novel thiol-containing natural products. Their C-S bonds are formed by oxidative coupling reactions catalyzed by EgtB and OvoA enzymes, respectively. In this work, it was discovered that besides catalyzing the oxidative coupling between histidine and cysteine (1 → 6 conversion), OvoA can also catalyze a direct oxidative coupling between hercynine (2) and cysteine (2 → 4 conversion), which can shorten the ergothioneine biosynthetic pathway by two steps.

Sulfur is an important functional group in both primary and secondary metabolites.¹ Biological sulfur transfer reactions can make use of either an ionic or a radical-type of reaction mechanism. For example, the thioether formation in lantibiotic biosynthesis is an ionic type of reaction that is accomplished by having thiolates as the direct nucleophiles.² Radical type C-S bond formation reactions are also well-documented.^{1a, 3} Some of the examples are: the incorporation of the thiol-functional groups into biotin,⁴ lipoate,^{3a} thiamine pyrophosphate,⁵ molybdopterin cofactor,⁶ cofactor biogenesis in galactose oxidase,⁷ tRNA nucleotide thiolmethylation,⁸ and thiazolidine ring formation catalyzed by isopenicillin *N* synthase.⁹ For sulfur transfer reactions, two types of activated sulfur species have also been discovered,¹⁰ which are persulfides R-S-SH and thiocarboxylates R-CO-SH.

Ergothioneine (5) and ovothiol (8) are two thiol-imidazole containing natural products. Humans obtain ergothioneine from their diet and specifically enrich it in some tissues (e.g. liver, kidney, central nervous system and red blood cells) by using an ergothioneine-specific

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Supporting Information Available Experimental procedure, characterization data and copies of the ¹H- and ¹³C-NMR of the synthesized compounds 2, 4 and 6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

transporter.¹¹ Ergothioneine's beneficial effects to human health are due to its unusual redox property, which favors predominantly the thione form (**5b**, Scheme 1A) and makes ergothioneine much more stable than most other natural thiols.^{1c-d,11g} Ergothioneine was isolated from ergot by Tanret in 1909,¹² and its biosynthetic gene cluster was reported by Seebeck in 2010 (Scheme 1A).¹³ Ergothioneine biosynthesis starts from histidine methylation to trimethylated histidine (hercynine, **2**), which is then oxidatively coupled with γ -glutamylcysteine (γ -Glu-Cys) to **3**. After glutamate is removed by hydrolysis, a PLP-containing enzyme (EgtE)-catalyzes the **4** $\rightarrow$ **5** conversion. Ovothiol is another thiol-histidine enriched in the eggs of many marine species. Ovothiol is proposed to be involved in H₂O₂ scavenging and facilitating the fertilization process¹⁴. For ovothiol biosynthesis (Scheme 1B), the only known enzyme is OvoA, which catalyzes the oxidative coupling between Cys and His (**1** $\rightarrow$ **6** conversion).¹⁵ Compare to ergothioneine, the C-S bond in ovothiol is at the δ instead of the ϵ position.

Both EgtB and OvoA are mononuclear non-heme iron enzymes catalyzing four-electron oxidation processes (Scheme 1). However, EgtB and OvoA distinguish themselves from each other by their substrate preferences and product C-S bond regio-selectivity (Scheme 1). Ergothioneine's thiol group is located at its imidazole ϵ -carbon, while ovothiol's thiol group is at its imidazole δ -carbon. In addition, EgtB and OvoA use different substrates. EgtB catalyzes the oxidative coupling between hercynine (**2**) and γ -Glu-Cys while OvoA preferentially oxidatively couples His and Cys. In this report, a key factor governing OvoA-catalysis regio-selectivity was discovered. By systematically modulating the histidine methylation state, the OvoA-catalysis changes from OvoA-type of chemistry (**1** $\rightarrow$ **6** conversion) to EgtB type of chemistry (**2** $\rightarrow$ **4** conversion). Such a discovery can shorten the ergothioneine biosynthetic pathway by two steps.

EgtB and OvoA were overexpressed in *E. coli* and purified anaerobically using Strep-tavidin affinity chromatography (Figure 1S). The purified proteins have close to stoichiometric amount of iron. To examine EgtB and OvoA substrate specificity, a ¹H-NMR assay was utilized (Figure 1). In ¹H-NMR spectrum, the chemical shifts of the EgtB product (**3**) imidazole H-atom, OvoA product (**6**) imidazole H-atom, and the substrate (histidine) are well-separated from the rest of the reaction mixture (Figure 1). Thus, the reaction mixture can be analyzed routinely without a need of the product separation (Figure 1). The signals with chemical shifts of 6.93 ppm and 7.70 ppm are assigned to the histidine imidazole H-atoms. The signal at 7.83 ppm is from the imidazole H-atom of ovothiol biosynthetic intermediate **6** (reaction D, Figure 1), while for the oxidative product **3** in ergothioneine biosynthesis, its imidazole H-atom has a chemical shift of 7.12 ppm (reaction A, Figure 1).

Using the ¹H-NMR assay, we systematically examined EgtB and OvoA substrate binding pocket flexibility (Scheme 2). EgtB does not accept OvoA's substrates as its alternative substrates (reactions B and C, Scheme 2). Interestingly, OvoA has much more flexibility in substrate specificity. OvoA can accept γ -Glu-Cys as a substrate and oxidatively couples it with His to form compound **15** (reaction E, Scheme 2). OvoA also can accept hercynine (**2**) as a substrate (reaction F, Scheme 2). More interestingly, ¹H-NMR spectrum of this reaction gives a new signal at 7.12 ppm (reaction F, Figure 1). In comparison with the ¹H-NMR spectra of native EgtB reaction (reaction A, Figure 1) and native OvoA reaction (reaction D,

Figure 1), the 7.12 ppm signal highly suggests that compound **4** is the product from the OvoA-catalyzed oxidative coupling between Cys and hercynine (Scheme 2, Figure 1C and Figure 11S). Formation of **4** implies a change in OvoA regio-selectivity from the OvoA-type (reaction D, Figure 1) to the EgtB-type (reaction F, Figure 1) upon the change of histidine to hercynine.

To provide further evidence to support compound **4** structural assignment, it was isolated and characterized by mass spectrometry and several NMR-spectroscopies (^1H -NMR, ^{13}C -NMR, and 2D-NMR including COSY, HMBC and HMQC, Figure 2 and Figures 12S-17S). ^1H - ^{13}C correlations between H-5 and C-4, C-6 in HMBC characterization supports compound **4** structural assignment (Figure 2). Additional cross-peaks (between H-3' and C-6, and between H-3 and C-4, C-5, Figure 2) are also consistent with the compound **4** structure. HMQC spectrum suggests that the only proton in compound **4** imidazole ring (δ 7.45 ppm) is at histidine δ -carbon (122 ppm, Fig. 16S), which provides additional evidence for an OvoA-catalyzed direct **2** $\rightarrow$ **4** conversion. In Figure 2 and Fig. 16S spectra, in order to well-resolve the resonances in the 3.0 – 4.5 ppm region, the sample is acidified. As a result, the imidazole ring hydrogen chemical shift (Figure 2) is now at 7.45 ppm instead of 7.12 ppm in Figure 1.

Upon the change of histidine to hercynine, OvoA changes its oxidative C-S bond formation regio-selectivity. This result highly suggests that OvoA binding pocket for histidine amino group plays a key role in determining imidazole ring binding orientation, which in turn determines the oxidative C-S bond formation regio-selectivity. To test this hypothesis, mono- and dimethyl-histidine were synthesized. Indeed, OvoA can also make use of them as alternative substrates. Interestingly, when monomethyl-histidine (**9**) and cysteine are used as the substrates, OvoA produces two oxidative coupling products, compound **10** and compound **11** in a ratio of 2:3 (Scheme 3 and Figure 19S). In case of dimethyl-histidine (**12**), compound **13** is the dominant product and compound **14** is barely detectable in our ^1H -NMR assay (Scheme 3 and Figure 20S). Results from these studies indicate that OvoA substrate binding pocket for the histidine amino group plays a key role in orientating substrates in the enzyme active site, which in turn determines the oxidative C-S bond formation regio-selectivity (Scheme 3).

In air-saturated HEPES buffer ($\sim 250\ \mu\text{M}$ of oxygen), these new OvoA reactions were characterized kinetically by monitoring the oxygen consumption rate using the NeoFoxy oxygen electrode. A) When histidine and cysteine are the substrates, kinetic parameters are: k_{obs} of $572 \pm 20\ \text{min}^{-1}$ and K_m of $420 \pm 31\ \mu\text{M}$ for His, K_m of $300 \pm 34\ \mu\text{M}$ for Cys; B) When Cys and monomethyl histidine (**9**) are the substrates, the kinetic parameters are: k_{obs} of $527 \pm 10\ \text{min}^{-1}$ and a K_m of $466 \pm 32\ \mu\text{M}$ for monomethyl histidine, a K_m of $0.99 \pm 0.05\ \text{mM}$ for Cys; and C) When Cys and dimethyl histidine (**12**) are the substrates, the kinetic parameters are: k_{obs} of $367 \pm 8\ \text{min}^{-1}$ and a K_m of $466 \pm 38\ \mu\text{M}$ for hercynine, a K_m of $1.61 \pm 0.12\ \text{mM}$ for Cys; D) When Cys and hercynine (**2**) are the substrates, the kinetic parameters are: k_{obs} of $270 \pm 5\ \text{min}^{-1}$ and a K_m of $395 \pm 30\ \mu\text{M}$ for hercynine, a K_m of $3.19 \pm 0.41\ \text{mM}$ for Cys.

In summary, our studies here revealed that OvoA has a very relaxed substrate binding pocket and can accept EgtB substrates as alternatives. Moreover, by modulating the histidine amino group methylation state, the regio-selectivity of OvoA-catalysis changes from the OvoA-type to the EgtB-type (Scheme 3). Due to many of the ergothioneine's beneficial roles to human health,^{1c, 16} there is a long interest of developing more efficient methods for its production.¹⁷ The discovery of this unique OvoA chemistry (a direct **2** → **4** conversion) suggests that such a chemistry may be further explored for future ergothioneine production through metabolic engineering. This transformation not only shortens the ergothioneine biosynthetic pathway by two steps (eliminate EgtA and EgtC steps, please referring to Scheme 1), but also eliminates the competition between ergothioneine and glutathione biosyntheses because γ -Glu-Cys is a substrate in both EgtB reaction and glutathione biosynthesis.

Several mechanistic models were proposed for the oxidative C-S bond formation in EgtB and Ovo-A catalysis since their discovery.^{15, 18} The oxidative C-S bond formation in OvoA and EgtB seems to be distinct from currently known biological C-S bond formation mechanisms.^{1a, 1c-d, 8} Thus far, mechanistic evidence is not yet available for differentiating among the proposed mechanistic options in the literature. Both EgtB and OvoA catalyze four-electron oxidation processes and two different functional groups are constructed in these reactions (sulfoxide and C-S bond, Scheme 4). Thus, the first mechanistic issue to be addressed in OvoA and EgtB catalysis is whether the sulfenic acid formation (model A) or the C-S bond formation (model B) is the first step. This mechanistic question is currently under investigation in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

¹H-NMR assay (chemical shifts of imidazole ring H-atoms). **Reaction A.** Native EgtB reaction in ergothioneine biosynthesis. The chemical shift assignments are: **3**: The imidazole H-atom of compound **3** (7.12 ppm); **2**: The hercynine imidazole H-atoms (6.85, 7.58 ppm). **Reaction D.** Native OvoA reaction in ovothiol biosynthesis. The chemical shift assignments are: **6**: The imidazole H-atom of compound **6** (7.83 ppm); **1**: The imidazole H-atoms of His (6.93, 7.70 ppm). **Reaction F.** A new OvoA reaction (oxidatively coupling between hercynine and Cys). In this case, the oxidative coupling product is compound **4**, which has the EgtB type of regio-selectivity. Reaction list is shown in Scheme 2.

Figure 2. HMBC-NMR analysis

of compound **4**. H-5 shows ¹H-¹³C correlations between C-4 (orange circle) and C-6 (cyan arrow). Additional cross-peaks between H-3' and C-6 (blue circle), H-3 and C-4 (green circle), C-5 (pink circle) are also shown.

Scheme 1.

Proposed ergothioneine and ovothiol biosynthetic pathways.

I. EgtB reaction list

II. OvoA reaction list

III. Product structures

Scheme 2.

Examining EgtB- and OvoA-substrate specificities.

Scheme 3.
New OvoA-chemistries.

Scheme 4.
Two OvoA mechanistic routes.