

Biochemical and Structural Characterization of OvoA_{Th2}: A Mononuclear Nonheme Iron Enzyme from *Hydrogenimonas thermophila* for Ovothiol Biosynthesis

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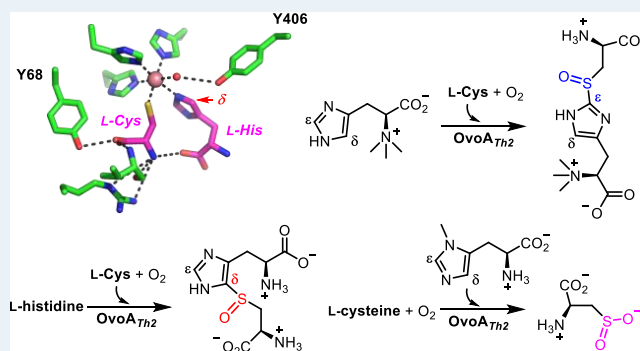
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ABSTRACT: Ovothiol A and ergothioneine are thiol-histidine derivatives with sulfur substitutions at the δ -carbon or ϵ -carbon of the L-histidine imidazole ring, respectively. Both ovothiol A and ergothioneine have protective effects on many aging-related diseases, and the sulfur substitution plays a key role in determining their chemical and biological properties, while factors governing sulfur incorporation regioselectivities in ovothiol and ergothioneine biosynthesis in the corresponding enzymes (OvoA, Egt1, or EgtB) are not yet known. In this study, we have successfully obtained the first OvoA crystal structure, which provides critical information to explain their C–S bond formation regioselectivity. Furthermore, OvoA_{Th2} exhibits several additional activities: (1) ergothioneine sulfoxide synthase activity akin to Egt1 in ergothioneine biosynthesis; (2) cysteine dioxygenase activity using L-cysteine and L-histidine analogues as substrates; (3) cysteine dioxygenase activity upon mutation of an active site tyrosine residue (Y406). The structural insights and diverse chemistries demonstrated by OvoA_{Th2} pave the way for future comprehensive structure–function correlation studies.

KEYWORDS: nonheme iron enzyme, ovothiol, ergothioneine, X-ray structure, regioselectivity, sulfur-containing natural products



INTRODUCTION

Ovothiol A (1) and ergothioneine (2) are naturally occurring thiol-histidine derivatives, featuring a sulfur substitution at the δ -carbon or the ϵ -carbon of their imidazole ring (Scheme 1),^{1,2} respectively. The locations of the sulfur atom within the imidazole side-chain of ovothiol A 1 and ergothioneine 2 resulted in different physical and biological properties.² The pK_a of ovothiol's sulfur (~ 1.4) is significantly more acidic than that of other natural thiols (usually falling within a range of 7.0 to 9.0).^{2–6} Ovothiol A inhibits cell proliferation due to its activation of an autophagic process in human hepatocarcinoma cell lines, Hep-G2,⁷ which indicates its potential anticancer activities. In contrast, ergothioneine is found primarily in its thiolate form under physiological conditions.^{8,9} Ergothioneine's reduction potential ($E_0' = -0.06$ V)^{6,10,11} is significantly higher than that of glutathione ($E_0' = -0.25$ V).¹² Ergothioneine can efficiently eradicate reactive oxygen species (ROS) and reactive nitrogen species (RNS).^{13–15} Moreover, ergothioneine possesses protective effects in several human diseases, such as rheumatoid arthritis,^{16,17} Crohn's disease,^{18,19} neurodegenerative diseases,^{20–23} cardiovascular disorders,²⁴ diabetes,²⁵ and fatty liver disease.^{26,27} Ergothioneine may also cross the blood-brain barrier,^{20–22} to protect

against neurodegenerative diseases. Notably, Ames recently hypothesized that ergothioneine might be a longevity vitamin.²⁸

Besides their biological activities, the biosynthetic and mechanistic characterization of sulfur-containing natural products has sparked new chemistries and enzymology.^{29–32} While L-cysteine or methionine commonly serves as sulfur sources in sulfur-containing peptides, the realm of sulfur incorporation chemistry extends far beyond peptide-based natural products. Exemplifying this versatility is ovothiol A and ergothioneine biosynthesis, which present elegant instances across various facets,^{1,30} encompassing sulfur sources, aerobic vs anaerobic biosynthetic routes, exploration of diverse catalytic strategies, meticulous control over sulfur-transfer regioselectivity, and notably, the shared and distinct features between sulfur and selenium chemistries.^{33–36} Over the past

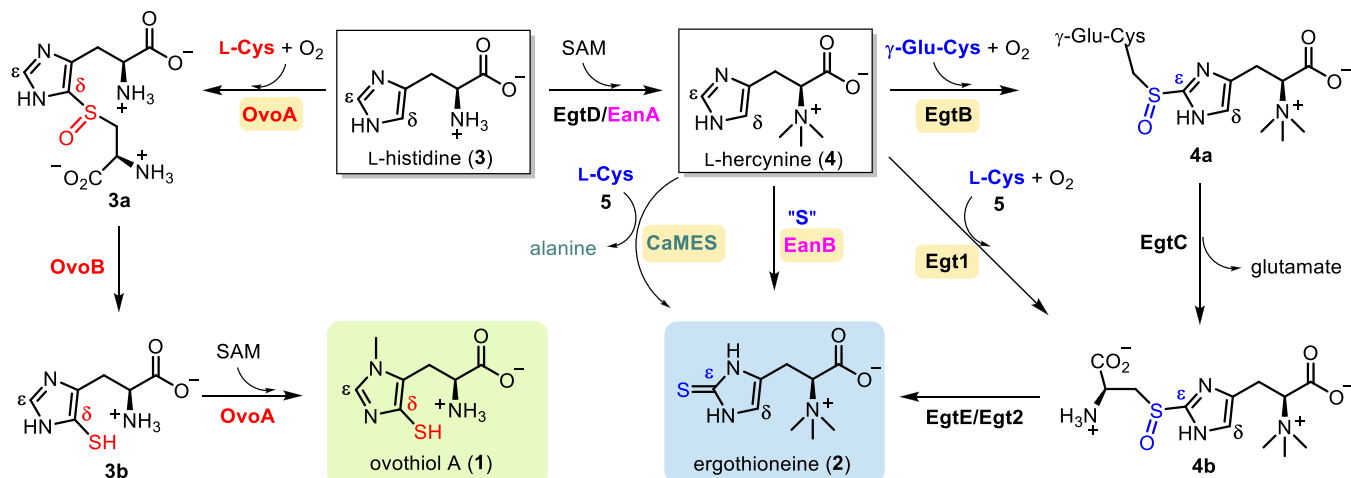
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Scheme 1. Biosynthetic Pathways of Ergothioneine and Ovothiol A^a

^aThe four ergothioneine biosynthetic routes are depicted on the right, while the aerobic ovothiol A biosynthetic pathway is illustrated on the left. Crucial C–S bond construction steps are catalyzed by EgtB, Egt1, EanB, CaMES, and OvoA, respectively.

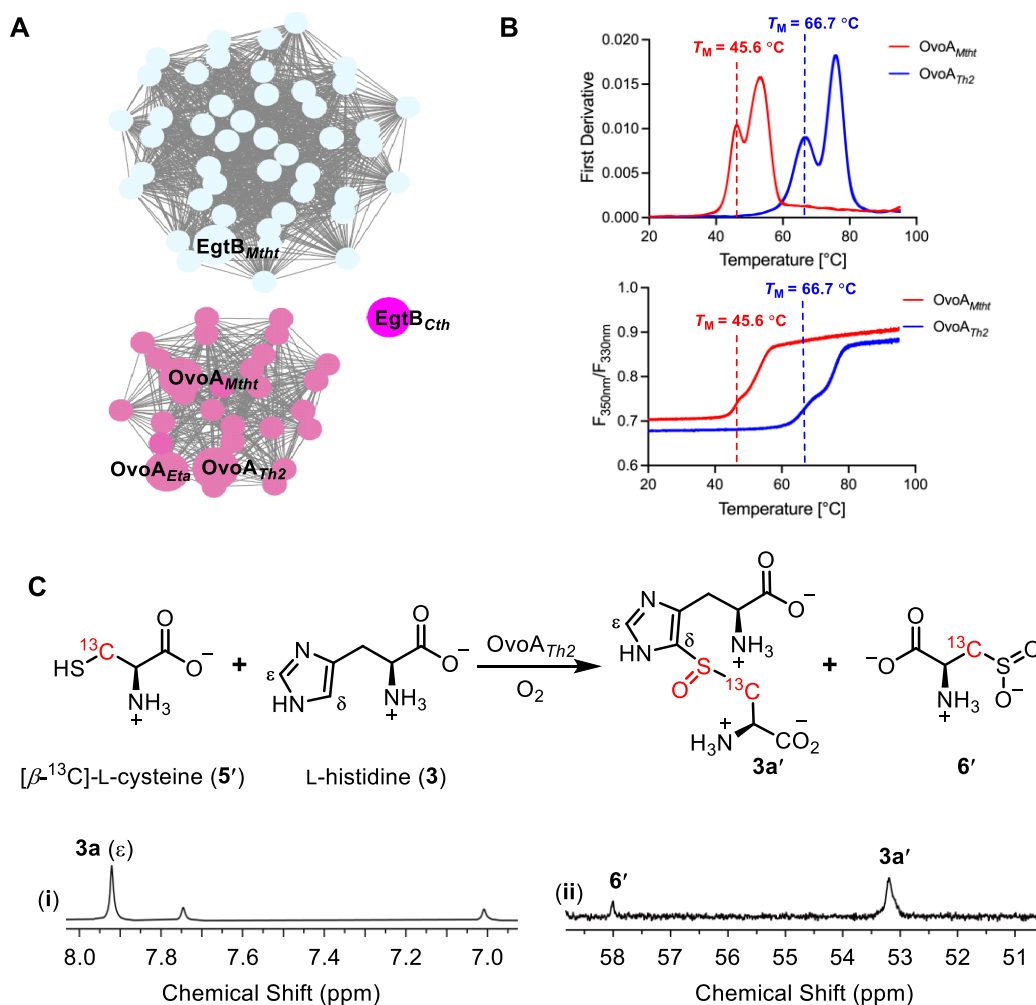


Figure 1. OvoA_{Th2} characteristics. (A) Sequence similarity network analysis with an *E*-value of 10⁻⁶⁰, highlighting EgtB and OvoA nodes; (B) assessment of thermostability for OvoA_{Mtht} and OvoA_{Th2} via NanoDSF; (C) OvoA_{Th2}-catalyzed reactions using L-histidine and L-cysteine/[β-¹³C]-L-cysteine substrates, obtained through ¹H NMR and ¹³C NMR analysis.

decade, four biosynthetic pathways for ergothioneine and one for ovothiol A have been elucidated. In the two aerobic pathways leading to ergothioneine, namely the mycobacterial

pathway (4 → 4a → 4b → 2 transformations catalyzed by EgtB, EgtC, and EgtE enzymes, Scheme 1)^{37,38} and the fungal *Neurospora crassa* pathway (4 → 4b → 2 transformations

Table 1. Kinetic Parameters of Wild-Type OvoA_{Th2} and Its Mutants under Different Conditions

enzyme	substrates	k_{cat, O_2} [min ⁻¹]	K_M , L-His or L-His derivatives [μM]	K_M , L-Cys [μM]	K_M , L-His or L-His derivatives or L-Cys [min ⁻¹ μM ⁻¹]	k_{cat}/K_M , L-Cys [min ⁻¹ μM ⁻¹]	% of coupling product from NMR analysis
wild-type	L-His + L-Cys	589.0 ± 3.5	585.1 ± 28.0	278.6 ± 9.0	2.10 ± 0.09		~90%
wild-type	L-Her + L-Cys	127.2 ± 1.5	61.9 ± 3.5	(1.86 ± 0.04) E3	66.9 ± 2.3		~50%
wild-type	L-Cys	26.7 ± 0.4		(2.6 ± 0.2) E3			below the detection limit
wild-type	3-Me-L-His + L-Cys	131.2 ± 2.7	1.9 ± 0.1	252.2 ± 14.5	0.52 ± 0.04		below the detection limit
wild-type	1-Me-L-His + L-Cys	20.5 ± 0.3		(3.9 ± 0.1) E3	(5.3 ± 0.1) E-3		below the detection limit

enzyme	substrates	k_{cat, O_2} [min ⁻¹]	K_M , L-His [μM]	K_M , L-Cys [μM]	k_{cat}/K_M , L-Cys [min ⁻¹ μM ⁻¹]	% of coupling product from NMR analysis
Y406F	L-His + L-Cys	361.2 ± 5.9	38.2 ± 2.6	575.2 ± 22.0	0.63 ± 0.09	~20%
Y68F	L-His + L-Cys	23.4 ± 0.2	50.9 ± 2.5	(61.9 ± 1.5) E3	(0.4 ± 0.1) E-3	~30%
Y68F/Y406F	L-His + L-Cys	14.8 ± 0.3	31.3 ± 4.0	(125.7 ± 11.0) E3	(1.2 ± 0.1) E-4	~10%

catalyzed by Egt1 & Egt2/EgtE enzymes, Scheme 1),^{39,40} pivotal C–S bond formation steps are catalyzed by mononuclear nonheme iron enzymes. In particular, **4** → **4a** is catalyzed by EgtB, and **4** → **4b** is catalyzed by Egt1, using γ -glutamyl-cysteine (γ -GC)³⁷ or L-cysteine³⁹ as the sulfur sources, respectively. Subsequently, a PLP-dependent C–S lyase orchestrates the **4b** → **2** transformation catalyzed by either EgtE or Egt2 (Scheme 1), finalizing the sulfur transfer from L-cysteine to the L-histidine side-chain.^{38,40} Recently, two anaerobic ergothioneine biosynthetic pathways have come to light. In the pathway of the green sulfur bacterium *Chlorobium limicola* (**3** → **4** → **2** transformations catalyzed by EanA-EanB catalysis, Scheme 1), the sulfur transfer is mediated by a rhodanese-domain-containing protein, EanB, utilizing polysulfide as the direct sulfur source and L-cysteine persulfide as an intermediate.^{41,42} In the *Caldithrix abyssi* ergothioneine biosynthetic pathway of involving **3** → **4** → **2** transformations (Scheme 1), a molybdenum-containing enzyme, CaMES,⁴³ is responsible for the vital sulfur transfer step. While L-cysteine has been suggested as the sulfur source, the mechanistic intricacies of CaMES catalysis are yet to be thoroughly investigated. Currently, a solitary ovothiol A biosynthetic pathway has been characterized (**3** → **3a** → **3b** → **1** transformations catalyzed by OvoA and OvoB enzymes, Scheme 1).^{44,45} In this ovothiol biosynthetic pathway, OvoA is a bifunctional enzyme encompassing sulfoxide synthase activity (**3** → **3a**, Scheme 1) and methyltransferase activity (**3b** → **1** transformations catalyzed by OvoA enzyme, Scheme 1).⁴⁴ Concurrently, OvoB serves as a PLP-dependent C–S lyase.⁴⁵

Over the past decade, a pivotal query in the realm of ergothioneine and ovothiol biosynthetic research involves unraveling the factors that dictate the regioselectivity of these nonheme iron enzymes (EgtB/Egt1 vs OvoA).⁴⁶ Recently, two X-ray crystal structures of ergothioneine sulfoxide synthases, namely EgtB_{Mthr}⁴⁷ and EgtB_{Ctho}^{48,49} have emerged. However,

no crystal structure of the OvoA has been documented thus far. This report addresses this gap in knowledge by presenting the biochemical and structural insights of an ovothiol sulfoxide synthase, OvoA_{Th2}, derived from *Hydrogenimonas thermophila*. This is the first crystal structure of an ovothiol sulfoxide synthase.

RESULTS AND DISCUSSION

Identification of the Ovothiol Biosynthetic Enzymes Derived from a Thermophilic Organism, *H. thermophila*.

Due to the sulfur atom's importance in determining the chemical and biological properties of ovothiol A and ergothioneine, there has been extensive interest in uncovering the factors governing the sulfoxide synthase regioselectivities, i.e., OvoA vs EgtB/Egt1 selectivity.⁴⁶ The biochemically characterized OvoA isolated from *Erwinia tasmaniensis* (OvoA_{Eta}),^{44,46,50,51} is very labile, and all crystallization efforts failed. Using OvoA_{Eta} as the query sequence, we searched for the homologues in thermophiles or mesophilic microorganisms from UniProt and NCBI databases.⁵² In total, 230 homologues were obtained. Using a protein sequence similarity network analysis approach, at an *E*-value cutoff of 10⁻⁶⁰, seventy-five representative sequences were categorized into two clusters (the EgtB branch and the OvoA branch), as well as a separate enzyme EgtB_{Cth} carrying both Egt1 and EgtB activities (Figure 1A).^{48,53} Among those sequences found within the OvoA node is the *H. thermophila* (WP_092912839) sequence was intriguing due to the optimal growth temperature of the microbe of approximately 55 °C.⁵⁴ This protein was subsequently named OvoA_{Th2}. Analysis of the protein domain organization of OvoA_{Th2} suggests that similar to that of OvoA_{Eta}, OvoA_{Th2} has both sulfoxide synthase and methyltransferase domains (Figure S1). Due to thermostability concerns in OvoA crystallization, after overexpression and purification of OvoA_{Th2} (Figure S2) using a protocol similar to

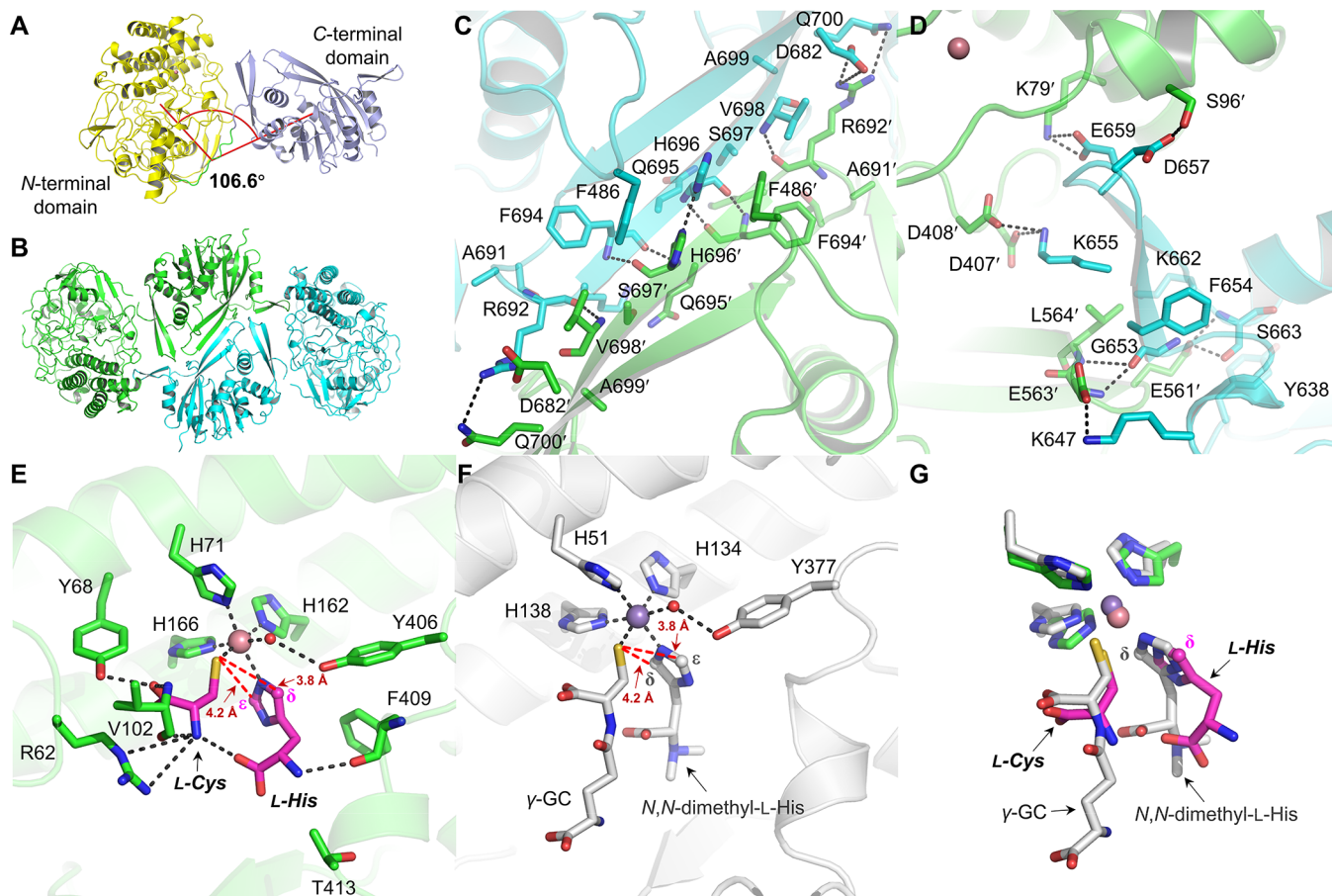


Figure 2. Crystal structure of $OvoA_{Th2}$. (A) Domain arrangement of $OvoA_{Th2}$. The $OvoA\cdot Co^{II}\cdot L\text{-Cys}\cdot L\text{-His}$ complex (PDB ID: 8KHQ) is shown in cartoon mode with yellow and light blue colors to present the *N*-terminal and *C*-terminal domains with a green linker, respectively. (B) Overall architecture of the $OvoA_{Th2}$ homodimer. The two monomers are colored green and cyan. (C and D) Dimer interfaces of $OvoA_{Th2}$. Each monomer was colored green and cyan, and the interacting residues were labeled with polar interactions highlighted in black dashes. (E) The active site of the $OvoA\cdot Co^{II}\cdot L\text{-Cys}\cdot L\text{-His}$ ternary complex. The residues around the active site in the *N*-terminal domain were shown as green sticks, while the metal cobalt as a pink sphere. (F) Active site of $EgtB\cdot Mn^{II}\cdot \gamma\text{-GC}\cdot N,N\text{-dimethyl-L-His}$ ternary complex (PDB ID: 4 × 8D).⁴⁷ The residues around the active site were shown as gray sticks, while the metal manganese as a sphere. (G) Superimposition between $EgtB\cdot Mn^{II}\cdot \gamma\text{-GC}\cdot N,N\text{-dimethyl-L-His}$ complex and $OvoA\cdot Co^{II}\cdot L\text{-Cys}\cdot L\text{-His}$ complex. The C_{δ} of the *L*-histidine imidazole ring was shown in sphere.

those employed for $OvoA_{Eta}$ and $OvoA_{Mtht}$ characterization,^{46,52} we assessed the thermostability of $OvoA_{Th2}$ using a nanoDSF assay (Figure 1B).^{55,56} The thermal unfolding curves of the peptide of $OvoA_{Mtht}$ are included for comparison purposes. Our analysis demonstrated that the T_M of $OvoA_{Th2}$ is approximately 21 °C higher than that of $OvoA_{Mtht}$ (Figure 1B).

Domain analysis of $OvoA_{Th2}$ suggested that it possesses an *N*-terminal formylglycine-generating enzyme (FGE) sulfatase domain as well as a *C*-terminal methyltransferase domain (Figure S1). $OvoA_{Eta}$ contains a similar domain organization, with our biochemical characterization supporting the bifunctionality of $OvoA_{Eta}$: ovothiol sulfoxide synthase and methyltransferase (Scheme 1).⁴⁵ $OvoA_{Th2}$ catalysis was analyzed using three different assays to provide evidence to support the activity of $OvoA_{Th2}$ as an ovothiol sulfoxide synthase (Figures 1C and S3): (a) ¹H NMR to examine the *L*-histidine imidazole side-chain for ovothiol sulfoxide synthase regioselectivity; (b) ¹³C NMR assay for *L*-cysteine activities using [β -¹³C]-*L*-cysteine as a substrate; (c) oxygen consumption rate analysis utilizing a Neo-Foxy oxygen electrode to assess the overall O₂ consumption rate.

In the ¹H NMR spectra, based on previously reported results pertaining to $Egt1/EgtB/OvoA$,^{39,46,52} the ~7.60 and 6.80

ppm peaks were assigned to *L*-histidine imidazole H_ε and H_δ, respectively. In the $OvoA_{Th2}$ catalyzed reaction, when *L*-histidine (3) and *L*-cysteine (5) were utilized as substrates, the product ¹H NMR signal was found to be ~7.92 ppm (trace i, Figures 1C and S3), assigned to H_ε based on prior studies on $OvoA_{Eta}$ ⁴⁶ which suggests that $OvoA_{Th2}$ catalyzes sulfoxide formation at the δ-position, the ovothiol class of sulfoxide regioselectivity. In the ¹³C NMR spectrum of the reaction mixture from the *L*-histidine and [β -¹³C]-*L*-cysteine reaction, two signals at 58.0 and 53.2 ppm were observed (trace ii, Figure 1C). The 53.2 ppm signal is consistent with [β -¹³C]-sulfoxide product 3a' as reported in prior $OvoA_{Eta}$ studies.^{52,57} The signal at 58.0 ppm is consistent with *L*-cysteine sulfinic acid,⁵⁷ while the level of [β -¹³C]-*L*-cysteine sulfinic acid 6' represents approximately ~10% of the product mixture. Therefore, $OvoA_{Th2}$ indeed acts as an ovothiol sulfoxide synthase. The oxygen consumption assay using a NeoFoxy electrode indicated that the kinetic parameters of $OvoA_{Th2}$ using *L*-histidine and *L*-cysteine at 23 °C are $k_{cat, O_2} = 589.0 \pm 3.5 \text{ min}^{-1}$; $K_M, L\text{-His} = 585.1 \pm 28.0 \mu\text{M}$, and $K_M, L\text{-cys} = 278.6 \pm 9.0 \mu\text{M}$ (Table 1 and Figure S4).

Overall Structure of $OvoA_{Th2}$. After demonstrating that $OvoA_{Th2}$ is the ovothiol sulfoxide synthase for ovothiol

biosynthesis, we sought to crystallize it. Because OvoA_{Th2}'s thermal unfolding temperature T_M is ~ 21 °C higher than that of OvoA_{Mth} (Figure 1B), our crystallization efforts on OvoA_{Th2} indeed were successful, obtaining the OvoA_{Th2} crystal structure at a resolution of 2.7 Å in complex with its two substrates, L-histidine and L-cysteine (Figure 2, OvoA·Co^{II}·L-Cys·L-His, PDB ID: 8KHQ). OvoA_{Th2} contains two functional domains: a sulfatase domain at the N-terminus (7–455) and a SAM-dependent methyltransferase domain at the C-terminus (466–707). Each domain forms a distinctive globular region, and these two domains are linked via a linker loop (456–465). The shaft angle between the mass centers of the two domains and the center of the linker is 106.6° (Figure 2A). Two OvoA_{Th2} monomers dimerize through a 2-fold symmetry in which the two C-terminal methyl transferase domains are at the center, flanked by the two N-terminal domains on either side. Such an arrangement results in a flat, rhomboid-shaped structure with three nodes (dimer of C-terminal domains in the middle, two N-terminal domains on each side) (Figure 2B). Based on calculations using the PISA server,⁵⁸ the dimer interface of OvoA_{Th2} is 1,951 Å², occupying approximately 6.5% of the total surface accessible area. Two OvoA_{Th2} regions are responsible for the dimerization. The first region is a β -strand resulting from residues T690–Q700 at the C-terminus. The β -strands from the two monomers stack in an antiparallel manner. Moreover, salt bridges were observed to link R692 of one monomer to D682' of the other monomer. The H696 residue forms a hydrogen bond with the same residue H696' on the other monomer while interacting with F486' through π stacking, which may help to orient the C-terminal domains (Figure 2C). The second monomer interface is primarily driven by the C-terminal domain of one monomer with the N-terminal domain of the other monomer. Residues G653–K662 form a short β -hairpin, which slots into the cavity built from N78' to S100' and I405' to D408' from the N-terminal domain of the other monomer (Figure 2D). The residues flanking this β -hairpin, (i.e., Y638–W650 and S663–D665) also participate in dimerization through interactions with the β -hairpin formed by T560'–L564' in the other monomer. Apart from hydrophobic interactions, intensive polar interactions also occur, including salt bridging between K647:E563', K655:D407', K655:D408', and E659:K79', as well as hydrogen bonding between D657:S96', S663:E561', and G653:E563', G653:L564' (Figures 2D and S5).

Active Site Analysis of the OvoA_{Th2}·Co^{II}·L-Cys·L-His Ternary Complex. Upon cocrystallization of OvoA_{Th2} with its substrates, L-cysteine and L-histidine, we mixed apo OvoA_{Th2}, Co^{II}, L-Cys, and L-His in a molar ratio of 1:3:3:3 and successfully solved the structure of OvoA_{Th2}·Co^{II}·L-Cys·L-His complex, which was crystallized anaerobically in an anaerobic Coy-chamber to avoid transformations during the crystallization process (Figures 2E and S6). The metallo-center is coordinated by three L-histidine residues (H71, H162, and H166). The L-histidine substrate coordinates to Co^{II} *trans* to H71 using the imidazole N1 atom, and the L-cysteine substrate is *trans* to H162. The sixth coordination site is occupied by a water molecule (outlined in chain B), which is a potential oxygen-binding site. A conserved tyrosine residue (Y406) forms a hydrogen bond with the coordinating water at a distance of 3.1 Å (visible in chain B, Figure 2E). The L-cysteine substrate is also involved in a hydrogen bonding network involving R62, Y68, the backbone of V102, and the carboxyl group of the L-histidine substrate. Apart from interactions with

the L-cysteine substrate, the L-histidine substrate also interacts with the F409 backbone by hydrogen bonds, and a portion of L-histidine is also close to Y406 as well as the nonpolar portion of T413 through nonpolar contacts (Figure 2E).

To examine structural factors crucial for the sulfoxide formation regioselectivity in OvoA_{Th2} relative to ergothioneine sulfoxide synthase (Egt1 or EgtB), we focused on the OvoA_{Th2}N-terminal domain as it is the sulfoxide synthase domain. EgtB from *Mycobacterium thermophilum* (EgtB_{Mthr}, PDB ID: 4 × 8D)^{47,49} possesses the highest structural similarity to OvoA_{Th2}, with an RMSD of 2.3 Å. EgtB_{Mthr} uses *N,N,N*-trimethyl-histidine (L-hercynine) and γ -glutamyl-cysteine (γ -GC) as substrates (Scheme 1).^{37,47,59} Both ergothioneine and ovothiol sulfoxide metallo-centers are coordinated by four imidazoles of L-histidine/hercynine residues (three L-histidine residues from the protein and one from the L-histidine/hercynine substrate) and one L-cysteine, leaving an additional site unoccupied for O₂ binding and activation. Seebeck and co-workers successfully solved the crystal structure of EgtB_{Mthr}·Mn^{II}· γ -GC·*N,N*-dimethyl-L-histidine complexes (Figure 2F).⁴⁷ In the OvoA_{Th2}·Co^{II}·L-Cys·L-His and EgtB_{Mthr}·Mn^{II}· γ -GC·*N,N*-dimethyl-L-histidine complexes (Figure 2G), the L-cysteine portions possess a similar orientation. Interestingly, the imidazole of the *N,N*-dimethyl-L-histidine in the EgtB_{Mthr}·Mn^{II}· γ -GC·*N,N*-dimethyl-L-histidine complexes flips approximately 180° relative to the L-histidine in the OvoA_{Th2}·Co^{II}·L-Cys·L-His complex. Such differences expose the L-histidine C δ to the L-cysteine sulfur atom in the OvoA_{Th2}·Co^{II}·L-Cys·L-His complex, while in the EgtB_{Mthr}·Mn^{II}· γ -GC·*N,N*-dimethyl-L-histidine complex, the C ϵ position in *N,N*-dimethyl-L-histidine is closer to the cysteine sulfur atom in γ -GC (Figure 2G). In our prior biochemical characterizations, we have demonstrated that the use of *N,N*-dimethyl-L-histidine and L-hercynine as OvoA_{Eta} substrates result in the same sulfoxide synthase regioselectivity, the ergothioneine type.⁴⁶ Therefore, this comparative structural analysis suggests that the relative orientation of the L-histidine imidazole side-chain in the enzyme active site determines the ovothiol and ergothioneine sulfoxide synthase regioselectivities.

The largest difference between the active site of OvoA_{Th2} and EgtB_{Mthr} is a loop region (residues 98 to 117 in OvoA_{Th2} vs residues 80–95 in EgtB_{Mthr}), acting as a lid to cover the subpocket for the L-cysteine substrate. In EgtB_{Mthr}, this loop region flips toward the solvent (Figure S7). We proposed that this loop region modulates the substrate preference in this family of sulfoxide synthases, where the relatively smaller subpocket in OvoA covered by this loop could accommodate only L-cysteine as the sulfur donor. At the same time, the more spacious pocket in EgtB_{Mthr} could accommodate γ -GC as the sulfur donor. Similar conclusions can also be drawn from the comparison of the crystal structure of EgtB_{Cth} from *Chloracidobacterium thermophilum* to OvoA_{Th2}.^{48,49} Residues 91 to 111 in EgtB_{Cth} represent the loop serving as the lid for covering the subpocket.

In EgtB_{Mthr} a tyrosine residue (Y377) has been proposed to be critical to the sulfoxide synthetase activity.⁴⁷ In EgtB_{Cth},^{48,49} two tyrosine residues (Y92 and Y93) are catalytically important. In OvoA_{Th2}, there is a conserved tyrosine residue (Y406) in the same position relative to Y337 in EgtB_{Mthr}. Given the similarity between OvoA_{Th2} and EgtB_{Mthr}, Y406 in OvoA_{Th2} may be critical for ovothiol sulfoxide synthase activity.

Functional Diversities of OvoA_{Th2} Derived from *H. thermophila*. In two OvoA enzymes reported in the literature

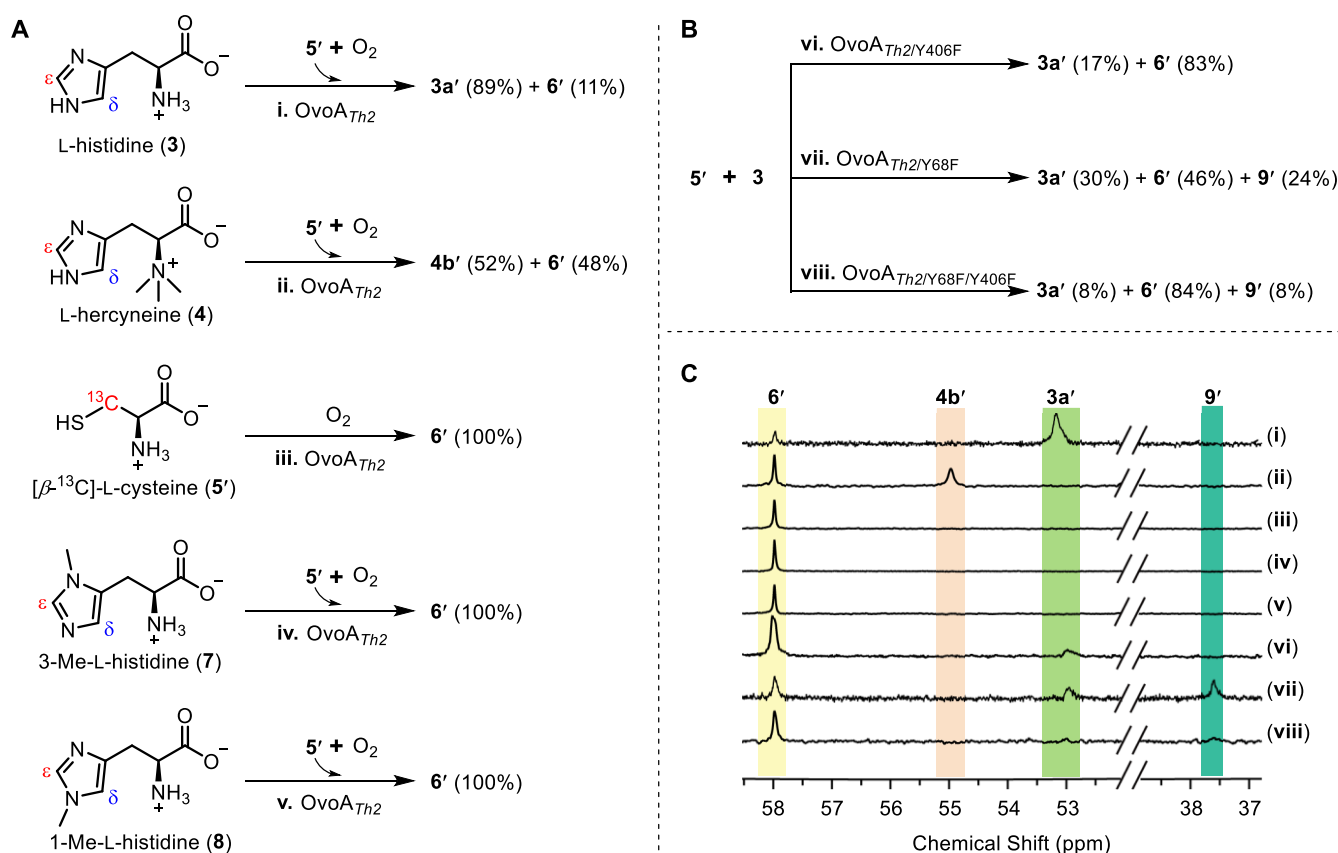


Figure 3. Characterization of *OvoA_{Th2}* and its variants under a few different conditions. (A) Results of wild-type *OvoA_{Th2}*-catalyzed reactions using [β-¹³C]-L-cysteine (5') and L-histidine (3) or derivatives (L-hercyrine (4), 3-methyl-L-histidine (7), and 1-methyl-L-histidine (8)) as substrates or using 5' as the only substrate. (B) Evaluation of *OvoA_{Th2}* variants *OvoA_{Th2/Y406F}* and *OvoA_{Th2/Y68F}* and its double mutant *OvoA_{Th2/Y68F/Y406F}* for their relative sulfoxide and CDO-like synthase activity using 5' and 3 as substrates. (C) ¹³C NMR analysis of reactions: wild-type *OvoA_{Th2}* using 5' and 3 as substrates (i), *OvoA_{Th2}* using 5' and 4 as substrates (ii), *OvoA_{Th2}* using 5' as the only substrate (iii), *OvoA_{Th2}* using 5' and 7 as substrates (iv), *OvoA_{Th2}* using 5' and 8 as substrates (v), *OvoA_{Th2/Y406F}* using 5' and 3 as substrates (vi), *OvoA_{Th2/Y68F}* using 5' and 3 as substrates (vii), and *OvoA_{Th2/Y68F/Y406F}* using 5' and 3 as substrates (viii).

(*OvoA_{Eta}*^{46,57,60} and *OvoA_{Mtht}*⁵²), outside of the ovothiol sulfoxide synthase activities, under some conditions, these enzymes produce L-cysteine sulfinic acid as a dominant product, displaying cysteine dioxygenase (CDO) activity. The partitioning between CDO and ovothiol sulfoxide synthase activities can be tuned through metallo-center ligands or by the secondary coordination shell residues. Critically, both *OvoA_{Eta}* and *OvoA_{Mtht}* have ergothioneine sulfoxide synthase activity when using L-hercyrine and L-cysteine as substrates.^{46,52} In the previous section, we summarized the crystal structure data from *OvoA_{Th2}*. The next key point to be addressed is whether the unique biochemical properties reported in the literature for *OvoA_{Eta}* and *OvoA_{Mtht}* are common to *OvoA_{Th2}*. If *OvoA_{Th2}* possesses the same properties, the structural information reported for *OvoA_{Th2}* will be a solid foundation for future structure–function correlation studies.⁶¹ To answer this question, we systematically characterized *OvoA_{Th2}* reactivity under various conditions (Figure 3 and Table 1): (1) ovothiol vs ergothioneine sulfoxide synthase activity; (2) cysteine dioxygenase activity; (3) roles of active site tyrosine residues.

OvoA_{Th2} is an ovothiol sulfoxide synthase (Figure 1). When [β-¹³C]-L-cysteine was used to replace cysteine, it generated [β-¹³C]-sulfoxide 3a' as a major product and [β-¹³C]-L-cysteine sulfinic acid 6' as a minor product (~10% of the reaction mixture). Interestingly, *OvoA_{Th2}* possesses ergothio-

neine sulfoxide synthase activity (Figure S8). Upon swapping in L-hercyrine for L-histidine, ¹H NMR clearly indicated that *OvoA_{Th2}* is an ergothioneine sulfoxide synthase, as demonstrated by the signal at 7.23 ppm in the ¹H NMR spectrum, which is the signal for the imidazole δ-position hydrogen atom (Figure S8). This result suggests sulfoxide formation at the L-hercyrine ε-carbon. This conclusion was further supported by ¹³C NMR analysis of the reaction using L-hercyrine and [β-¹³C]-L-cysteine as substrates. In the ¹³C NMR spectrum, the signal at 55.0 ppm was derived from [β-¹³C]-sulfoxide 4b', and the signal at 58.0 ppm was derived from [β-¹³C]-L-cysteine sulfinic acid 6'. The ratio between 4b' and 6' is approximately 1:1 (trace ii, Figure 3). Kinetic analysis using an O₂ consumption assay demonstrated that the kinetic parameters for this reaction at 23 °C are $k_{cat, O_2} = 127.2 \pm 1.5 \text{ min}^{-1}$; $K_M, L-Her} = 61.9 \pm 3.5 \mu\text{M}$, and $K_M, L-Cys} = 1.86 \pm 0.04 \text{ mM}$ (Figure S9, Table 1). Clearly, upon changing L-histidine to L-hercyrine, *OvoA_{Th2}* makes a corresponding alteration in activity from an ovothiol sulfoxide synthase to an ergothioneine sulfoxide synthase. We measured the CDO activity of the molecule of *OvoA_{Th2}* using L-cysteine as the substrate. The signal at 58.0 ppm in the ¹³C NMR experiments did support the CDO activity when L-cysteine was the only substrate (trace iii, Figures 3 and S10). The steady-state kinetics parameters are $K_M, L-Cys} = 2.6 \pm 0.2 \text{ mM}$, and a much lower $k_{cat, O_2} = 26.7 \pm 0.4 \text{ min}^{-1}$ (Figure S11, Table 1).

The alterations in activity upon the replacement of L-histidine with L-hercynine clearly suggest that the catalysis of OvoA_{Th2} is sensitive to subtle modifications to the enzyme's active site. We examined two additional histidine analogs, 3-methyl-L-histidine (7) and 1-methyl-L-histidine (8), which have the two imidazole nitrogen atoms methylated, respectively. Using 3-methyl-L-histidine (7) and L-cysteine as substrates, only $[\beta\text{-}^{13}\text{C}]$ -L-cysteine sulfinic acid 6' (δ_{C} 58.0) was observed as a product (trace iv, Figures 3 and S12–S13, Table 1) with the $k_{\text{cat}, \text{O}_2} = 131.2 \pm 2.7 \text{ min}^{-1}$. Similar results were also observed when 1-methyl-L-histidine (8) and L-cysteine were used as substrates (trace v, Figures 3 and S14, Table 1), producing only $[\beta\text{-}^{13}\text{C}]$ -L-cysteine sulfinic acid 6' with $k_{\text{cat}, \text{O}_2} = 20.5 \pm 0.3 \text{ min}^{-1}$ (Figure S15).

Results from the above experiments demonstrated that the activities of the OvoA_{Th2} can be tuned across ovoidiol sulfoxide synthase, ergothioneine sulfoxide synthase, and CDO activities. When L-histidine and L-cysteine were substrates, ~90% of the product in the reaction mixture was found to be sulfoxide 3a. However, in the case of L-hercynine and L-cysteine reactions, ergothioneine sulfoxide 4b and L-cysteine sulfinic acid 6 are produced at comparable levels. In the cases of both 3-methyl-L-histidine and 1-methyl-L-histidine, OvoA_{Th2} displays CDO activity, with reduced k_{cat} values by ~4.5 and 28.7-fold, respectively (Table 1).

In the structure of the $\text{OvoA}_{Th2}\cdot\text{Co}^{\text{II}}\cdot\text{L-Cys}\cdot\text{L-His}$ complex, two tyrosine residues, Y68 and Y406, are close to the metallo-center and substrate binding site. It has been identified that active site tyrosine residues are critical to ovoidiol and ergothioneine sulfoxide synthase activity.^{48–52,60} We, therefore, characterized the phenylalanine mutants of these two tyrosine residues. Three mutants have been biochemically and kinetically analyzed, including Y68F and Y406F single mutants and the Y68F/Y406F double mutant of OvoA_{Th2} . The kinetic parameters of these mutants are summarized in Table 1.

In the Y406F mutant, the kinetic parameters were measured through an O_2 consumption assay at 23 °C (Table 1 and Figures S16–S17). In comparison to the parameters obtained for wild-type OvoA_{Th2} , $\text{OvoA}_{Th2}/\text{Y406F}$ has a K_{M} for the L-histidine substrate decreased by 15-fold from $585.1 \pm 28.0 \mu\text{M}$ in wild-type OvoA_{Th2} to $38.2 \pm 2.6 \mu\text{M}$, while their k_{cat} s are comparable. The product analysis of the Y406F reaction determined that the dominant product (>80%) of the Y406F mutant reaction is $[\beta\text{-}^{13}\text{C}]$ -L-cysteine sulfinic acid 6', with a small amount of $[\beta\text{-}^{13}\text{C}]$ -sulfoxide 3a' representing ~20% of the product mixture (trace vi, Figure 3). In the Y68F mutant, the results differ from both the wild-type and Y406F mutant (Table 1 and Figures S18–S19). Compared to the parameters of the wild-type OvoA_{Th2} , the K_{M} for the L-cysteine substrate increases by nearly 200-fold, and the $k_{\text{cat}, \text{O}_2}$ decreased by approximately 25-fold for $\text{OvoA}_{Th2}/\text{Y68F}$. As a result, in the OvoA_{Th2} Y68F mutant, the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$, L-Cys) decreases by nearly 5.5×10^3 -fold. ^1H NMR and ^{13}C NMR analysis of the Y68F mutant reaction demonstrated that the product ratio of $[\beta\text{-}^{13}\text{C}]$ -sulfoxide 3a', $[\beta\text{-}^{13}\text{C}]$ -L-cysteine sulfinic acid 6', and $[\beta\text{-}^{13}\text{C}]$ -L-cystine 9' is approximately 5:3:2 (trace vii, Figures 3 and S18). Based on the crystal structure depicted in Figure 2, Y68 directly interacts with the carboxylate of the L-cysteine substrate through hydrogen bonding. In the double mutant variant Y68F/Y406F, the kinetic parameters are comparable to those of the Y68F mutant. However, ^1H NMR and ^{13}C NMR analysis suggested that the double mutant variant produces L-cysteine sulfinic acid

as the dominant product, while sulfoxide and cystine are below the detection levels (Table 1, trace viii in Figures 3 and S20–21). Therefore, with the use of 3-methyl-L-histidine and 1-methyl-L-histidine as the substrate or by using the Y68F/Y406F mutant, OvoA_{Th2} exhibits predominantly the CDO activity rather than an ovoidiol sulfoxide synthase activity. Such flexibility in the OvoA_{Th2} activities suggests it is an excellent platform for future structure–function relationship studies.

CONCLUSIONS

Sulfur is one of the most abundant elements in the natural world. In recent years, an interdisciplinary approach has been employed to characterize sulfur incorporation chemistry,³⁰ encompassing biosynthetic sulfur sources, intermediates, and, notably, mechanistic insights into new strategies for sulfur-related biosynthetic pathways. With the exponential expansion of microbial genomes accessible through public databases,⁶² new biosynthetic and mechanistic knowledge has been harnessed in genome mining initiatives to systematically identify related natural products and their underlying biosynthetic routes. In the biosynthesis of sulfur-containing natural products, L-cysteine, or its derivatives (e.g., glutathione and mycothiol), have conventionally served as direct sulfur sources. Recent instances include the use of thiocysteine in leinamycin biosynthesis⁶³ and the utilization of polysulfide in a specific anaerobic ergothioneine biosynthetic pathway.^{41,42} In numerous other cases, the precise sulfur sources remain unidentified and are simply denoted as "S".^{1,29,30,61}

Mechanistically, sulfur transfer chemistry can be accomplished through nucleophilic substitution or radical mechanisms. For nucleophilic substitution chemistry, the two common intermediates are thiocarboxylate or L-cysteine persulfide on carrier proteins, as evidenced in biosynthetic studies of BE-7585A.⁶⁴ For sulfur carrier proteins generating thiocarboxylate intermediates, they terminate with a C-terminal Gly-Gly. Some exceptions to this general structural motif have been reported, in which the C-terminal Gly-Gly motif was released from the carrier protein after proteolysis through the removal of the C-terminal residues by a dedicated protease.^{65,66} Recently, persulfides and thiocarboxylates have been implicated as key intermediates in the biosynthesis of thioamides, functional groups in sulfur-modified tRNA, and many small molecular natural products.³¹ Similar to thiazoline/thiazole in peptide-based natural products,⁶⁷ thioamide generation is generally an ATP-dependent process involving either phosphorylation or adenosylation activation³² and L-cysteine, protein-bound thiocarboxylates, or L-cysteine persulfides have been suggested as sulfur sources, while in many cases, the sulfur sources are unknown.³¹

In addition to these novel sulfur natural product biosynthetic examples, ergothioneine and ovoidiol A biosynthesis represent exceptional examples (Scheme 1). Their important biological properties are consistent with the use of several different strategies to produce these two compounds.²⁸ Both aerobic and anaerobic biosynthetic pathways involving sulfur's nucleophilic chemistries^{41–43} and radical chemistries¹ have been reported for ergothioneine biosynthesis. For the aerobic ergothioneine and ovoidiol A biosynthetic pathways, the crucial transformation is the nonheme iron enzyme-catalyzed sulfoxide formation reactions (EgtB, Egt1, and OvoA). One of the most important mechanistic questions is how these enzymes modulate the sulfoxide formation regioselectivities

(Scheme 1). Over the past decade, two X-ray crystal structures of ergothioneine sulfoxide synthases, namely EgtB_{Mthr}⁴⁷ and EgtB_{Cthv}^{48,49} have been reported. However, due to thermostability issues faced in the OvoA studies, it has been difficult to obtain the ovothiol sulfoxide synthase structure. In this work, using enzymes with significantly improved thermostability (~21 °C increase in thermostability for OvoA_{Th2} relative to OvoA_{Mtht}), we have obtained the first ovothiol sulfoxide synthase crystal structure. This paves the way for more detailed structure–function relationship studies.⁶¹ More importantly, in spite of the low sequence homology (47.6% identity relative to OvoA_{Mtht}) and a significant increase in thermostability, OvoA_{Th2} shares nearly all biochemical properties with OvoA_{Mtht}.⁵² First, OvoA_{Th2} possesses both ovothiol and ergothioneine sulfoxide synthase activities, and such tuning-in activities are controlled by the orientation of the imidazole side chain in the enzyme active site (Figure 2). Second, OvoA_{Th2} has CDO activity, and the activities between CDO and sulfoxide synthase can be modulated using either substrate analogs (e.g., 3-methyl-L-histidine or 1-methyl-L-histidine) or by mutations in active site tyrosine residues (e.g., Y68F/Y406F double mutant). The structural information reported in this study and the rich chemistries displayed by OvoA_{Th2} have laid a solid foundation for future structure–function correlation studies. This structural information may also assist future engineering efforts in obtaining enzymes that are more suitable for the production of ergothioneine and ovothiol in an industrial setting.⁶⁸

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.3c04026>.

Fermentation and compound purification conditions; enzymatic reactions under various combinations and under different conditions; NMR data for substrates, substrate analogs, and products from the enzymatic reactions; and additional crystal structural information (PDF)

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Author Contributions

#X.W., S.H., J.W., and T.Z. contributed equally to this work. P.L., X.L., W.Y., and L.Z. designed the study. S.H., T.Z., A.W., and X.W. performed biochemical experiments. W.Y., J.W., X.W., and K.Y. performed crystallization and structural determination. P.L., X.L., W.Y., L.Z., G.Z., and A.V. analyzed data and wrote the manuscript with input from all authors. All authors have given approval for the final version of the manuscript.

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Notes

The authors declare no competing financial interest. The structure of the OvoA_{Th2}-Co^{II}-L-Cys-L-His ternary complex (PDB ID: 8KHQ) can be accessed at <https://www.pdbus.org/>.

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