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Identification and Characterization of the First Ovothiol Biosynthetic Enzyme

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Supporting Information

ABSTRACT: Ovothiols are histidine-derived thiols that were first isolated from marine invertebrates. We have identified a 5-histidylcysteine sulfoxide synthase (OvoA) as the first ovothiol biosynthetic enzyme and characterized OvoAs from *Erwinia tasmaniensis* and *Trypanosoma cruzi*. Homologous enzymes are encoded in more than 80 genomes ranging from proteobacteria to animalia.

Ovothiol A (**1**, Figure 1), B, and C are π-*N*-methyl-5-thiohistidines with a very acidic thiol group (pK_a = 1.4),[1] proficiency as a one-electron donor,[2] and a redox potential (−0.09 V vs NHE) rivaling that of protein disulfide isomerases.[1,3] These properties render ovothiols efficient scavengers of radicals and peroxides with possible roles in the redox defense of a number of organisms.[2,4−6] For example, sea urchin eggs contain millimolar concentrations of ovothiol C, which protect the egg content during oxidative envelope maturation.[7] More recent reports that pathogenic *Trypanosoma* and *Leishmania* produce **1** raised the interest in its biochemistry and biosynthesis.[4,5,8] Cell-free extracts from *Crithidia fasciculata* revealed that assembly of **1** starts with the conversion of cysteine and histidine to a 5-histidylcysteine sulfoxide conjugate (**2**) in an oxygen-dependent reaction. This intermediate is then trimmed to 5-thiohistidine and methylated at the imidazole ring (Figure 1).[4,9,10]

Insertion of a sulfur atom into a nonelectrophilic, aromatic C−H bond is quite unusual, and the involvement of oxygen suggests a departure from known mechanisms for enzymatic C−S bond formation, which are either oxygen-independent or oxygen-sensitive.[11−13] Therefore, deciphering the enzymology of oxidative sulfur transfer presents an important challenge. Also, the physiological functions of **1** are poorly understood, and the potential of ovothiol biosynthesis as a target for anti-infective agents is untested. To enable such research, we have characterized the first ovothiol biosynthetic enzyme from *Erwinia tasmaniensis* and *Trypanosoma cruzi*.

We previously identified a mycobacterial enzyme (EgtB) that inserts the sulfur atom of γ-glutamylcysteine into the C2−H bond of histidine betaine to form an intermediate of the ergothioneine biosynthetic pathway (**3**, Figure 1).[14] On the basis of the similarity of this reaction with the first step in ovothiol biosynthesis, we surmised that distant EgtB homologues encoded in genomes of ovothiol producers[5] might be 5-histidylcysteine sulfoxide synthases (OvoAs). In agreement with this idea, all five sequenced trypanosomatid genomes contain genes with significant similarity to EgtB from *Mycobacterium smegmatis* ($E < 3 \times 10^{-7}$). EgtB and the putative OvoA share a domain with homology to formylglycine-generating enzymes and an uncharacterized N-terminal domain. In addition, the trypanosomatid protein contains a C-terminal putative methyltransferase domain that appears to be specific for ovothiol biosynthesis because ergothioneine-producing organisms lack proteins with significant homology. Using this additional domain as a criterion to distinguish OvoAs from EgtBs, we identified more than 80 OvoA homologues, predominantly from proteobacteria but also from uni- and multicellular eukaryotes (Figure S2 in the Supporting Information).

For in vitro characterization, we prepared recombinant OvoA from *E. tasmaniensis*[15] ($OvoA_e$) and *T. cruzi* ($OvoA_t$). Production in *Escherichia coli* and purification on Ni-NTA agarose yielded 5 mg/L $OvoA_e$ but less than 0.5 mg/L $OvoA_t$ (Figures S3−S5). To assess the catalytic activity, $OvoA_e$ was incubated with 1 mM histidine, 3 mM cysteine, 2 mM TCEP, 0.1 mM $FeSO_4$, 20 mM Tris (pH 8.0), and 20 mM NaCl at 26 °C. Cation-exchange HPLC analysis of this reaction revealed consumption of histidine and production of a new compound that was identified as **2** by mass spectrometry and NMR spectroscopy (HRMS: calcd. 291.07584, found 291.07577; Figures S6 and S7). A single proton signal in the aromatic range (C2−H: s, δ 8.1) is consistent with attachment of the sulfoxide group to the imidazol C5. To confirm this interpretation, we repeated the above experiment using C2−^{2}H histidine as the substrate and found that the corresponding product retained the isotopic label (HRMS: calcd 292.08204, found 292.08215; Figure S7). The preference for C5 versus C2 oxidation is the prime difference between OvoA and EgtB. Unlike **1**, the 2-thioimidazol ring of **3** exists predominantly in its thione form, which is less acidic ($pK_{a,SH}$ > 10), less nucleophilic, and markedly less prone to auto-oxidation than **1**.[16] It is quite likely that these differences translate into distinct biological roles for **1** and **3**.

On the basis of HPLC analysis, we determined that $OvoA_e$ produces **2** with a rate constant of 1.9 ± 0.2 min^{-1}, catalyzing at least 140 turnovers per active site (Figures S8 and S9). In comparison, the trypanosomal enzyme is significantly less active and less stable (Figure S10). The in vitro activities of both enzymes are modest, which might point to suboptimal assay conditions. For example, cysteine and/or histidine may not be the true substrates. Therefore, we assayed $OvoA_e$ with numerous thiols, such as glutathione and γ-glutamylcysteine, and with π-*N*-methylhistidine, α-*N*-methylhistidine, and α-*N*,*N*-dimethylhistidine but found no superior substrates (Figures S11−S15). Other factors, such as missing

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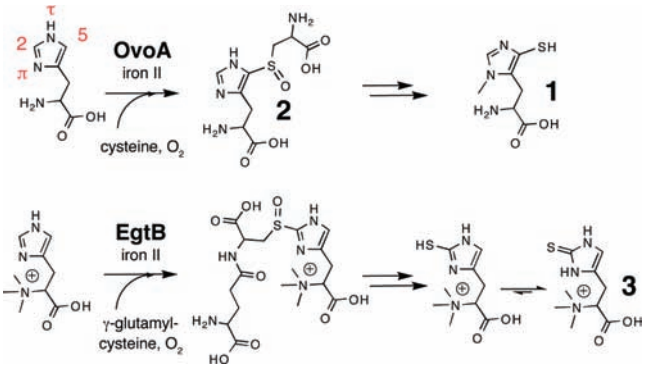

Figure 1. Biosynthesis of ovothiol A (**1**) and ergothioneine (**3**). We have adopted IUPAC histidine nomenclature to name ovothiol derivatives (in red). Ovothiols B and C are the α-*N*-methyl and α-*N*,*N*-dimethyl derivatives of ovothiol A; OvoA and EgtB are sulfoxide synthases involved in ovothiol and ergothioneine biosynthesis.

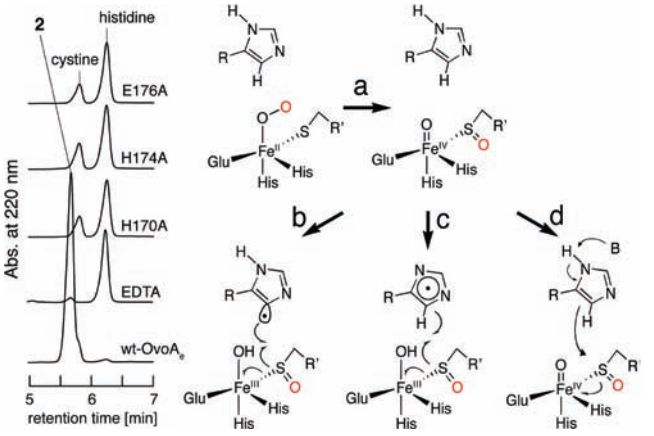

Figure 2. (left) HPLC analysis of enzymatic reactions of wild-type $OvoA_e$, iron-depleted wild-type $OvoA_e$, and $OvoA_e$ variants H170A, H174A, and E176A. HPLC signals were identified by ESI-MS. (right) Proposed mechanisms for OvoA: C−S bond formation is initiated by (b) formation of a histidyl sp^2 radical, (c) formation of a histidyl π-radical, and (d) electrophilic attack of the iron-coordinated cysteine sulfoxide on histidine.

post-translational modifications, absent protein complex partners, or suboptimal pH and buffer composition, may limit the in vitro activities of the recombinant enzymes.

OvoA and EgtB are both iron-dependent enzymes (Figure 2 and Figure S16).[14] The molecular basis for iron recognition probably maps to a conserved HX_3HXE motif in the N-terminal domains of both OvoA and EgtB (Figure S17). Consistent with this thought, we found that removing any one of the three side chains of His170, His174, and Glu176 reduces the $OvoA_e$ activity by at least 100-fold (Figure 2). Although 2-His-1-carboxylate facial triads are common iron-binding motifs,[17] the proximity of the ligands in the primary sequence of OvoA is unusual. An alternative protein fold or an atypical geometry at the iron center may necessitate this compact arrangement.

One further puzzle is why OvoA and EgtB couple C−S bond formation to sulfoxidation,[10,14,18] a modification that is not necessary for subsequent biosynthetic steps and is absent in the final product. Possibly, OvoA oxidizes cysteine to access an iron(IV)−oxo state (a in Figure 2) which then mediates oxidative sulfurization of histidine. Similarly, many non-heme iron enzymes depend on α-ketoglutarate as an electron donor.[19] For example, taurine hydroxylase oxidizes α-ketoglutarate to succinate and carbon dioxide to produce an iron(IV)−oxoiron species that abstracts a hydrogen atom from a methylene group of taurine.[20] The resulting carbon radical recombines with an iron-bound hydroxyl radical to form hydroxytaurine. An analogous OvoA mechanism would require the formation of an sp^2 radical (b in Figure 2). Such species are highly unstable, making hydrogen atom transfer a good candidate for the rate-limiting step. Despite this expectation, we could not detect any kinetic isotope effect in a competition between histidine and C-2,5,α-^{2}H-histidine for $OvoA_e$-catalyzed turnover (Figure S18). Clearly, more experimental work is necessary to examine this mechanistic proposal and investigate alternative pathways, such as via the formation of a histidyl π-radical or via electrophilic attack of iron-coordinated cysteine sulfoxide on histidine (c and d, respectively, in Figure 2).

This report has characterized OvoA, the first enzyme in ovothiol biosynthesis. Our data show that the enzyme requires coordination of iron(II) to an unusual iron-binding motif. Cysteine and histidine are preferred $OvoA_e$ substrates, but the modest in vitro activities may suggest that the recombinant enzymes are not fully active. To our knowledge, OvoA is the first characterized protein that mediates histidine side-chain modification at C5. The identification of OvoA sets the stage for the construction of ovothiol-deficient trypanosomatids to test whether this biosynthetic pathway is a target for novel anti-infective therapeutics.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S18, Table S1, detailed experimental procedures, and ^{1}H and 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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