The enzyme catalyzes the first step in ovothiol A biosynthesis. This enzyme sulphurizes the C5 position of the imidazole side chain of L-histidine. We report the substrate specificity profile of this catalyst and present data which indicate that OvoA catalysis follows an thiol-ene type mechanism.

Ovothiol A (1, Fig. 1) is a thiohistidine derivative which has been discovered in sea urchin eggs1–3 and human pathogens such as Leishmania major and Trypanosoma cruzi.4–7 Because of its thiol function ovothiol A is characterized by a remarkably low pK, of 1.4,8 and an increased redox potential (–0.09 V vs. SHE) compared to glutathione (–0.26 V)8 or trypanothione (–0.24 V).9 These distinct parameters suggest that ovothiol A occupies functional niches in cellular redox homeostasis but its precise physiological roles are unknown.4–7,10–12

Ovothiol A is biosynthesized from L-cysteine, L-histidine, molecular oxygen (O2) and S-adenosyl methionine (SAM) (Fig. 1).4–6 The key step in this pathway is oxidative insertion of a sulphur atom into the C2–H bond on the histidine side chain. Subsequent elimination of the L-cysteine derived carbon scaffold from intermediate 2 (Fig. 1) and reduction of the oxidized 5-thiohistidine complete the transfer of a sulphur atom from L-cysteine to L-histidine. Recently we characterized an iron(II) dependent enzyme, OvoA, from Erwinia tasmaniensis which mediates this unusual oxidative sulphur transfer.13 We also described an OvoA homolog from Mycobacterium smegmatis, EgtB, which is involved in ergothioneine biosynthesis (3, Fig. 1).14 The mycobacterial enzyme inserts a sulphur atom into the C2–H bond on the imidazole ring of γ-N,N,N-trimethyl-L-histidine (4, Fig. 1).

The catalytic mechanism and the substrate scope of this novel class of non-heme iron enzymes are poorly understood. In the present report we demonstrate that OvoA catalyzes efficient in vitro sulphurization of L-histidine, n-histidine, 2-fluoro L-histidine and compounds other than amino acids. In addition, we discuss indications that OvoA may catalyze C–S bond formation by a thiol-ene reaction mechanism,15 in which an OvoA generated L-cysteine thyl radical attacks the unsaturated imidazole ring of γ-L-histidine.

To initiate this study, we produced OvoA from E. tasmaniensis as previously reported.13 Typical OvoA reactions contained γ-cysteine, γ-histidine, 1 μM FeSO4, 1 mM ascorbate, 50 mM Tris HCl and 50 mM NaCl. The reactions were performed at 26 °C and were monitored by HPLC at 220 nm. Addition of either ascorbate or α-isoascorbate to the reaction mixture constituted a major improvement over our previous protocols15 because the antioxidants increased OvoA activity by nearly 100-fold (Fig. S1, ESI†). From a 800 μL reaction mixture containing 10 mg of OvoA, 1 mM γ-histidine and 1 mM γ-cysteine we were able to purify 100 mg of 5-γ-histidyl-γ-cysteine sulfoxide (2, Fig. 1, Fig. S2, ESI†),
demonstrating that OvoA is able to deliver thiolated \( \text{\textit{l}} \)-histidine at preparative scales.

OvoA catalysis is characterized by a \( k_{\text{cat}} \) of 3.3 s\(^{-1}\) and a \( k_{\text{cat}}/K_{\text{M}} \) of 9.4 \( \times \) 10\(^3\) M\(^{-1}\) s\(^{-1}\) and 1.0 \( \times \) 10\(^4\) M\(^{-1}\) s\(^{-1}\) for \( \text{\textit{l}} \)-cysteine and \( \text{\textit{l}} \)-histidine respectively (Table 1, Fig. S3, ESI†). The pH dependence of \( k_{\text{cat}}/K_{\text{M,his}} \) follows a bell shaped curve with an activity maximum at pH 7.3 flanked by the kinetic \( pK_a \)s 6.8 and 8.0 (Fig. S4, ESI†). Alkaline pH limits activity due to a declining \( k_{\text{cat}} \). At lower pH \( K_{\text{M,his}} \) becomes limiting. The lower kinetic \( pK_a \) coincides with the \( pK_a \) of \( \text{\textit{l}} \)-histidine, suggesting that OvoA binds this substrate in deprotonated form.

Using the same kinetic assay we then profiled the substrate specificity of OvoA. We previously determined that OvoA is highly specific for \( \text{\textit{l}} \)-cysteine as a sulphur donor and does not accept other thiols such as \( \text{\textit{d}} \)-cysteine, \( \text{\textit{l}} \)-histidine is not known, but studies on indoles and phenols suggest that fluorination can increase the redox potential of aromatic moieties by 60–200 mV.\(^{17,18}\) Despite these changes OvoA converts 2-fluoro-\( \text{\textit{l}} \)-histidine to 2-fluoro-5-\( \text{\textit{l}} \)-histidyl-\( \text{\textit{l}} \)-cysteine (24). Although an excellent \( \text{\textit{l}} \)-histidine isostere,\(^{16}\) 2-fluoro-\( \text{\textit{l}} \)-histidine presents a much more acidic (\( pK_a \approx 1 \)) and electron poor side chain.\(^{16}\) The redox potential of 2-fluoro-\( \text{\textit{l}} \)-histidine is not known, but studies on indoles and phenols suggest that fluorination can increase the redox potential of aromatic moieties by 60–200 mV.\(^{17,18}\) Despite these changes OvoA converts 2-fluoro-\( \text{\textit{l}} \)-histidine to 2-fluoro-5-\( \text{\textit{l}} \)-histidyl-\( \text{\textit{l}} \)-cysteine sulfoxide (30, HRMS: calcd 309.0669, found 309.0663) with almost the same efficiency as it converts \( \text{\textit{l}} \)-histidine to 2 (Fig. S8, ESI†).

The apparent insensitivity of OvoA to the redox potential of the sulphur acceptor calls our earlier attempts at explaining the underlying catalytic mechanism into question.\(^{13}\) These proposals (1–3, Fig. 3) predicted sulfoxidation of the substrate \( \text{\textit{l}} \)-cysteine as a requisite step to generate the enzyme bound oxo iron(IV) species (a) which then mediates C–S bond formation (b, c or d).\(^{13}\) This second step could proceed via homolytic cleavage of the imidazole \( C_\text{s}–H \) bond (b, mechanism 1). Because the resulting \( \text{sp}^2 \)
2-fluoro-L-histidine is a well-tolerated substrate. According to the imidazole ring serves as an electrophilic target for the state of the enzyme and concludes the catalytic cycle. Because subsequent sulfoxidation of the thioether restores the ferrous superoxide complex generates a L-cysteine thiyl radical which species.19 The absence of a significant solvent kinetic isotope effect (KIE solvent = 1.2 ± 0.1, Fig. S5, ESI†) and the observation that the electron poor 2-fluoro-L-histidine is an efficient OvoA substrate suggest that this step is either not rate limiting or does not occur. The third mechanism which implicates the imidazole ring as a nucleophile (d, mechanism 3) is similarly inconsistent with efficient turnover of 2-fluoro-L-histidine, and with the absence of a KIE solvent.

A fourth mechanism could explain the present observations more consistently: in this scheme the OvoA based iron(III)-superoxide complex generates a l-cysteine thyl radical which attacks the imidazole ring (e), followed by rearomatisation (f). Subsequent sulfoxidation of the thioether restores the ferrous state of the enzyme and concludes the catalytic cycle. Because the imidazole ring serves as an electrophilic target for the nucleophilic thyl radical it would not be surprising that 2-fluoro-l-histidine is a well-tolerated substrate. According to this mechanism C-S bond formation depends only on the presence of an unsaturated carbon on the sulphur acceptor. This scheme is reminiscent of the thiol-ene reaction which relies on the ability of photo-generated thyl radicals to attack olefins as a first step to thioether bond formation (Fig. 4).15 Given the broad scope of this reaction it seems possible that engineered or evolved sulfoxide synthases can be found which can sulphurize a broad range of unsaturated hydrocarbons.

The reported data present OvoA from E. tasmaniensis as an efficient catalyst allowing in vitro preparation of the sulphurized product (2, Fig. 1) on a 100 mg scale. The substrate specificity profile suggests that OvoA does not require an amino acid moiety on the sulphur acceptor. OvoA converts l-histidine into a mixture of C2 and C5 modified products indicating that product specificity is purely a function of substrate positioning in the active site. Finally, our observation that OvoA accepts 2-fluoro-l-histidine as an efficient substrate, coupled with the absence of KIE solvent or KIE substrate, point towards a catalytic mechanism which views the sulphur acceptor as the passive target of an iron(III)-superoxide generated thyl radical. In combination, these properties present OvoA as a promising scaffold for the engineering of tailor made sulphur transferases.

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Notes and references