Conversion of a non-heme iron-dependent sulfoxide synthase into a thiol dioxygenase by a single point mutation†

Kristina V. Goncharenko and Florian P. Seebeck*

EgtB from Mycobacterium thermoresistibile catalyzes O₂-dependent sulfur–carbon bond formation between the side chains of Nα-trimethyl histidine and γ-glutamyl cysteine as a central step in ergothioneine biosynthesis. A single point mutation converts this enzyme into a γ-glutamyl cysteine dioxygenase with an efficiency that rivals naturally evolved thiol dioxygenases.

Non-heme iron oxygenases catalyze a broad range of chemically difficult reactions and therefore provide intriguing starting points to develop novel enzymes for industrial applications. Despite remarkable progress in mechanistic enzymology,1–7 only few design studies describing modified non-heme iron enzymes with engineered activities8–12 have explored this potential for technological innovation so far. As a further step into this nascent field of enzyme engineering we present an example of a monooxygenase that was converted into an efficient thiol dioxygenase based on minimal active site redesign.13

The sulfoxide synthase (EC 1.14.99.50) EgtB catalyzes the central step in ergothioneine biosynthesis (1, Fig. 1).14–16 EgtB mediates carbon–sulfur bond formation between γ-glutamyl cysteine (γGC) and the imidazole ring of Nα-trimethylated histidine (TMH). Concomitant oxidation of the bridging sulfur atom (2, Fig. 1) makes the overall reaction a four-electron oxidation, and classifies EgtB as a monooxygenase.17 The active site of EgtB contains ferrous iron coordinated by a 3-His facial triad, the thiolate side chain of γGC and the imidazole ring of TMH (Fig. 2).16 In this structure the likely O₂-binding site is occupied by a water molecule or a hydroxide that also hydrogen bonds to the side chain of Gln55 (2.8 Å), a neighbouring water molecule (3.1 Å) and the side chain of Tyr377 (2.8 Å). In this report we show that substitution of Tyr377 to Phe completely changes the catalytic activity of EgtB.

The variant enzyme (EgtB<sub>Y377F</sub>) catalyzes dioxygenation of γGC with an efficiency similar to that of naturally evolved cysteine dioxygenases (CDO, EC 1.13.11.20).18,19 CDOs also bind ferrous iron by a 3-His facial triad combined with the thiolate and amine ligands from the substrate (Fig. 2), but the overall structures of EgtB and CDO are unrelated. A wealth of structural, biochemical, spectroscopic and computational investigations suggest that CDO catalysed formation of cysteine sulfinic acid (3) goes through a cysteine bound iron(III)–superoxo species (a, Fig. 2), followed by intermediates b and c.18–26 The structural and functional similarities between the active sites of CDO and EgtB raise the possibility that thiol dioxygenation and sulfoxide synthesis may proceed through at least one common catalytic intermediate.

The following kinetic analysis of EgtB<sub>wt</sub> and EgtB<sub>Y377F</sub> indicates that an iron(III)–superoxo species (A, a) may be this common intermediate.
To conduct this study we produced EgtB<sub>wt</sub> and EgtBY<sub>377F</sub> in <i>Escherichia coli</i>, and we monitored the rate of enzyme catalyzed sulfoxide (2) production using a published HPLC-based assay.<sup>16</sup> Michaelis–Menten analysis of this data revealed 10<sup>3</sup>-fold less sulfoxide synthase activity for EgtBY<sub>377F</sub> than for EgtB<sub>wt</sub>. This reduction is entirely due to a smaller k<sub>cat</sub> since K<sub>M,TMH</sub> remained unchanged (Table 1, Fig. S1 and S2, ESI†). We also determined the substrate kinetic isotope effect (KIE) using C<sub>2</sub>-deuterated TMH. Because both enzymes showed a substrate KIE near unity (Fig. S3, ESI†) we concluded that C<sub>2</sub>–H bond cleavage is not rate limiting in either enzyme and that hydrogen or proton removal from TMH is not an essential function of Tyr377.

When we compared the rates of γGC consumption and sulfoxide production (Fig. S4, ESI†) we found a rather surprising difference between the two enzymes. In EgtB<sub>wt</sub> catalyzed reactions, substrate consumption (k<sub>cat,wt</sub> = 0.4 ± 0.02 s<sup>−1</sup>) and sulfoxide formation (k<sub>sulfoxide,wt</sub> = 0.3 ± 0.06 s<sup>−1</sup>) were essentially coupled. By contrast, in EgtBY<sub>377F</sub> catalysed reactions, γGC consumption was much faster (k<sub>GC,Y<sub>377F</sub>=0.6 ± 0.1 s<sup>−1</sup>) than sulfoxide production (k<sub>sulfoxide,Y<sub>377F</sub></sub> = 0.002 s<sup>−1</sup>). NMR analysis of the completed reactions revealed that EgtBY<sub>377F</sub> oxidizes most substrate to γGC dioxide (4) instead (Fig. S5 and S6, ESI†). This new activity still depends on TMH, which indicates that dioxygenation proceeds via the same substrate complex as sulfoxide production. In the absence of TMH both EgtB<sub>wt</sub> and EgtBY<sub>377F</sub> catalyzed γGC dioxygenation at a similarly slow rate (k<sub>GC</sub> = 0.008 s<sup>−1</sup>, Fig. S7 and S8, ESI†). Apparently, the single substrate complex is significantly less reactive and its reaction specificity is not influenced by residue 377.

We also determined the Michaelis–Menten parameters for EgtBY<sub>377F</sub> catalyzed γGC dioxygenation (Table 1). Considering that

### Table 1: Kinetic parameters of EgtB variants

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>k&lt;sub&gt;cat,wt&lt;/sub&gt; (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;M,wt&lt;/sub&gt; (μM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;M&lt;/sub&gt; (M&lt;sup&gt;−1&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat,TMH&lt;/sub&gt; (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;M,TMH&lt;/sub&gt; (μM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;M&lt;/sub&gt; (M&lt;sup&gt;−1&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfoxide synthase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EgtB&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>8.0</td>
<td>7.5 × 10&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>27</td>
<td>2.8 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8.5 × 10&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>10</td>
<td>7.4 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>EgtBY&lt;sub&gt;377F&lt;/sub&gt;</td>
<td>8.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EgtB&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>6.0</td>
<td>1.1</td>
<td>370</td>
<td>2.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>22</td>
<td>5.7 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>EgtBY&lt;sub&gt;377F&lt;/sub&gt;</td>
<td>6.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>γGC dioxygenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EgtBY&lt;sub&gt;377F&lt;/sub&gt;</td>
<td>8.0</td>
<td>1.2</td>
<td>110</td>
<td>1.1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EgtBY&lt;sub&gt;377F&lt;/sub&gt;</td>
<td>6.0</td>
<td>0.9</td>
<td>320</td>
<td>2.7 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CDO&lt;sub&gt;murine&lt;/sub&gt;</td>
<td>7.5</td>
<td>1.8</td>
<td>700</td>
<td>2.6 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Standard deviation correspond to less than 20% of averaged value. Apparent k<sub>cat</sub> and k<sub>cat</sub>/K<sub>M</sub> in the presence co-substrate in a concentration at least 3-fold higher than the corresponding K<sub>M</sub>-
this protein may never have evolved to catalyze this alternative reaction it is particularly striking that its catalytic efficiency closely matches that of naturally evolved CDOs (Table 1).

The kinetic parameters for EgtB<sub>Y377F</sub> catalyzed dioxygenation are also remarkably similar to those for EgtB<sub>wt</sub> catalyzed sulfoxide synthesis (Table 1). Given the similar apparent <i>k<sub>a</sub></i> in both enzymes for both substrates we have no indication that the substitution of residue 377 affected binding of TMH or γGC. The fact that both enzymes oxidize γGC at similar rates further suggest that the efficiency of O<sub>2</sub> binding and activation have not changed. However, the resulting ternary complex (A, Fig. 2) does behave quite differently in the wild-type and variant protein.

In the presence of ascorbate EgtB<sub>wt</sub> and EgtB<sub>Y377F</sub> catalyze many hundreds of turnovers, without any sign of inactivation. Without ascorbate EgtB<sub>wt</sub> oxidizes to the inactive iron(III) form after approximately 100 turnovers, corresponding to an autoxidation rate of <i>k</i><sub>autoxidation</sub> = 0.01 s<sup>-1</sup> (Fig. S9, ESI†). This inactivation is reversible by addition of ascorbate, and is best explained by unproductive decay of the iron(III)-superoxide species A to superoxide and ferric EgtB<sub>Y377F</sub> inactivates 10-fold faster (<i>k</i><sub>autoxidation</sub> = 0.1 s<sup>-1</sup>, Fig. S10, ESI†), indicating that the initial iron coordinated oxygen species may be destabilized by the Tyr377 to Phe substitution (Fig. 2).

This substitution also influences the solvent KIE on EgtB. The sulfoxide synthase activity of EgtB<sub>wt</sub> and the γGC dioxygenase activity of EgtB<sub>Y377F</sub> are both characterized by a solvent KIE near unity (1.2 ± 0.2 and 0.9 ± 0.1, Fig. S11 and S12, ESI†). In contrast, the sulfoxide synthase activity of EgtB<sub>Y377F</sub> exhibited a solvent KIE of 1.9 ± 0.1 (Fig. S11, ESI†), indicating that one or multiple protons or hydrogen atoms are being transferred in the rate limiting step. Because the dioxygenase activity is not affected by solvent deuteration we conclude that this transfer occurs exclusively on sulfoxide synthase pathway. In the context of the proposed mechanisms for EgtB and CDO (Fig. 2) the most likely candidate for this solvent isotope sensitive step would be protonation of the iron(III)-superoxide intermediate A. Protonation of this oxygen species may be important to increase the thiyl radical character of the γGC ligand (B), which in turn could attack the imidazole ring of TMH (C). In EgtB<sub>Y377F</sub> the iron coordinated superoxide is not protonated but instead attacked the electron deficient sulfur atom on γGC (A to B). The analogous S-O bond forming step (a to b) has been found as the first irreversible step in CDO catalyzed cysteine dioxygenation (Fig. 2).<sup>16–26</sup>

According to this model, species A can either react via irreversible proton transfer to intermediate B, or via irreversible S-O bond formation leading to intermediate B′ simply depending on the availability of an acidic proton in the active site. We did indeed observe a 3.5-fold increase in <i>k<sub>a</sub></i> for EgtB<sub>Y377F</sub> catalyzed sulfoxide synthesis when the reaction pH was lowered from 8.0 to 6.0 (Table 1). The observed sulfoxide synthase activity is a hyperbolic function of proton concentration with a half-saturation point (<i>k<sub>a</sub></i><sub>proton</sub>) near pH 7 (Fig. S13, ESI†). This dependence is consistent with a general acid mechanism in which the phosphate buffer (pK<sub>a,monoanion</sub> = 7.2), or an alternative protein residue with a similar pK<sub>a</sub> can replace Tyr377 as an indirect proton source. The <i>k<sub>a</sub></i> of γGC dioxygenase activity of the same protein proved nearly constant in the same pH range (Table 1) which is in agreement with the proposition that acid catalysis is less important in the first irreversible step of thiol dioxygenation (A to B′ or a to b′).

In conclusion, we identified Tyr377 as a catalytic residue in EgtB<sub>Y377F</sub> from <i>M. thermoresistibile</i>. Mutation of this residue to Phe did not measurably affect substrate binding or O<sub>2</sub> activation, but instead changed the dominant activity of this enzyme. The remaining sulfoxide synthase activity of EgtB<sub>Y377F</sub> is characterized by an increased solvent KIE and significant dependence on buffer pH. These observations are best explained with a mechanistic model suggesting that (i) the sulfoxide synthase and the thiol dioxygenase reaction pathways share a common intermediate, (ii) that this intermediate is the iron(III)-superoxide species A, and (iii) that protonation by Tyr377 is essential to move this species towards sulfoxide synthesis, and away from γGC dioxygenation. This report the first example of a non-heme iron enzyme which could be engineered to efficiently catalyze a completely different reaction type than the parent enzyme.

CDOs and EgtB belong to entirely unrelated protein families and evolved along different selective pressures. The fact that EgtB<sub>Y377F</sub> catalyzes thiol dioxygenation with similar efficiency as CDOs makes the two enzymes a stunning example of accidental convergent evolution. We anticipate that detailed comparison of the two catalyst will prove a fruitful avenue to advance our current understanding of both reaction types.<sup>18–26</sup>

K. V. G. is a recipient of a Swiss Government Fellowship for Excellence; F. P. S. is supported by the “Professur für Molekulare Bionik” and by an ERC starting grant.

Notes and references