Supplementary information to:

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

Materials. All standard reagents were purchased from Aldrich/Sigma if not otherwise stated. Synthetic oligonucleotides were purchased from Microsynth, Switzerland.

Recombinant enzyme production. $EgtB_{wt}$ and $EgtB_{Y377F}$ were produced and purified following published protocols.[1] The gene for $EgtB_{Y377F}$ production was constructed by primer extension PCR using the following primers:

Y377Fs: 5'-TAT AGC CGA TGA TTT TTC AGC GCT ACA -3'
Y377Fa: 5'-TAT ATG TAG CGC TGA AAA ATC ATC GGC -3'
EgtBs: 5'-TAT ACA TAT GGG TGT CGC CGT GC -3'
EgtBa: 5'-ATA TCT CCG AGC TAA CAA CCA CCC ACC GG -3'

The resulting fragment was cloned into a pET28b vector. Purified $EgtB_{Y377F}$ contained 0.64 % equivalents of iron as inferred by a ferrozine-based colorimetric assay.[1] The extinction coefficient was calculated based on the primary sequence ($\varepsilon_{280,EgtBY377F}$: 109890 M^{-1} cm^{-1}).[2] The protein mass was determined by HRMS-ESI ($m/z_{EgtBY377F}$: calc.: 51326.67, meas.: 51358.226; Delta: 31.6 Da). A deviation of 32 Da between the calculated and the measured mass has also been observed for the wild type enzyme and is consistent with dioxygenation of the C-terminal cysteine residue.[1] At the present time we have no direct indication as to whether this modification is important for *in vitro* activity or the *in vivo* function of EgtB. Dioxygenation of exposed or activated cysteine residues has been reported for several protein before.[3] It is worth adding that EgtB from *Mycobacterium smegmatis* is characterized by similar *in vitro* catalytic parameters is EgtB from *M. thermoresistibile*, but lacks this 32 Da modification.

Michaelis-Menten analysis/Enzyme assay. Enzyme activities were assayed in 100 mM sodium phosphate buffer containing 100 mM NaCl, 2 mM TCEP, 2 mM ascorbate, 4 µM $FeSO_4$, TMH and enzyme (Figures 2a – h),. Reactions were started by addition of γGC and incubated at 26°C. Aliquots of the reactions were quenched by addition of 20 µL 1 M phosphoric acid and analyzed by cation exchange HPLC using 20 mM phosphoric acid at pH 2 as a mobile phase.[4] Compounds were eluted in a NaCl gradient. All HPLC chromatograms were recorded at 265 nm. The data were fitted to the function $v = V_{max}[s]/(K_M + [s])$. Michaelis-Menten plots are shown below. k_{cat} and k_{cat}/K_M were determined in the presence of the second substrate at a concentration at least 3-fold higher than K_M of the second substrate. The data displayed in Figures 2a – h corresponds to averages from three independent enzyme reactions.

γGC dioxygenase activity (Figures 2g – h), was quantified by monitoring consumption of γGC. Given that sulfoxide synthase activity in $EgtB_{Y377F}$ is 100 – 1000 fold less efficient than γGC dioxygenation we ignored the fact that a tiny fraction of γGC is converted to the sulfoxide

product. For HPLC based quantification of γGC the 40 µL reaction aliquots were quenched by addition of 40 µL acetonitrile and 10 µL of 20 mM 4-bromomethyl-7-methoxycoumarin (BMC) in DMSO. After incubation for 30 min the mixture was diluted with one volume equivalent of 0.1% TFA solution. Coumarin–adducts (Figure S1) were quantified by RP-HPLC by absorbance at 330 nm.

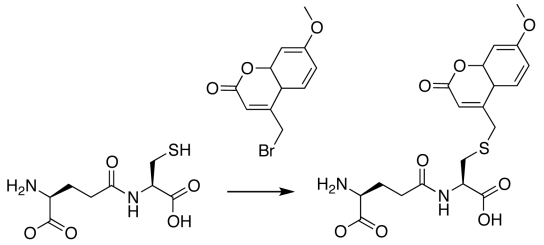

Figure S1. Formation of methyl-7-methoxycoumarin adduct of γGC

Figure S2a – h: Michaelis-Menten plots

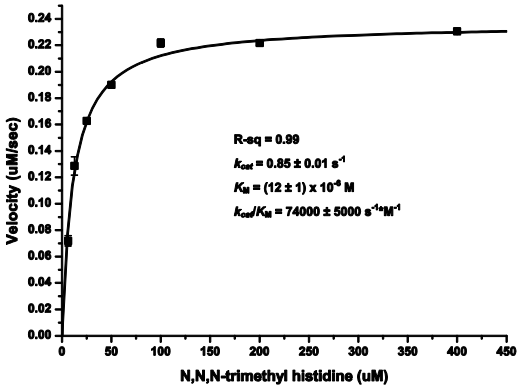

Figure S2a: phosphate buffer, pH 8.0 EgtB, [γGC] = 1200 µM

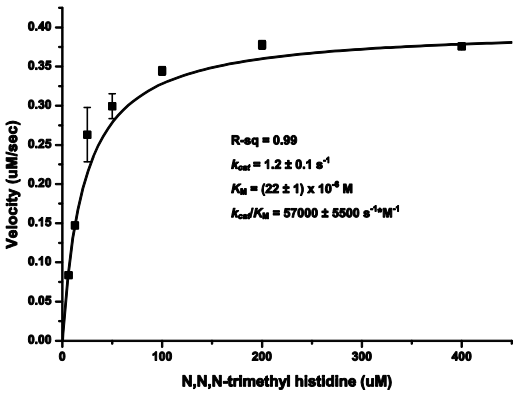

Figure S2b: phosphate buffer, pH 6.0 EgtB, [γGC] = 1200 µM

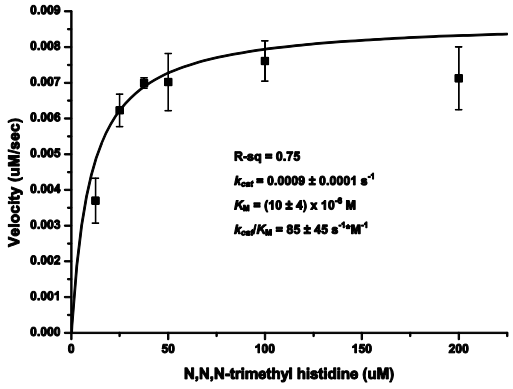

Figure S2c: phosphate buffer, pH 8.0 $EgtB_{Y377F}$, [γGC] = 1200 µM

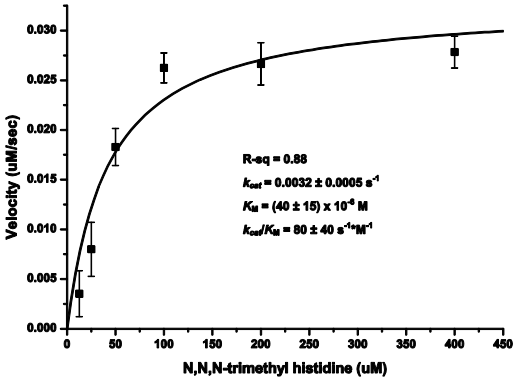

Figure S2d: phosphate buffer, pH 6.0 $EgtB_{Y377F}$, [γGC] = 1200 µM

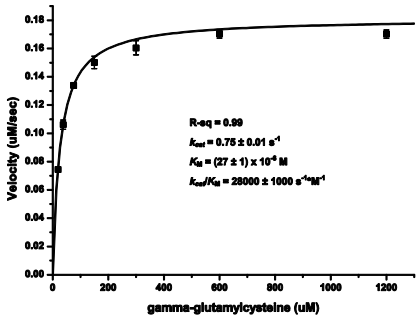

Figure S2e: phosphate buffer, pH 8.0 EgtB, [TMH] = 400 µM

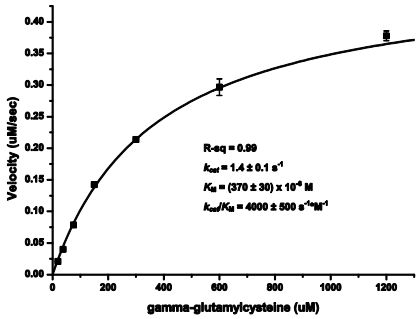

Figure S2f: phosphate buffer, pH 6.0 EgtB, [TMH] = 400 µM

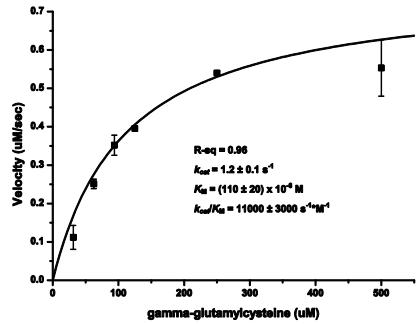

Figure S2g: phosphate buffer, pH 8.0 $EgtB_{Y377F}$, [TMH] = 400 µM

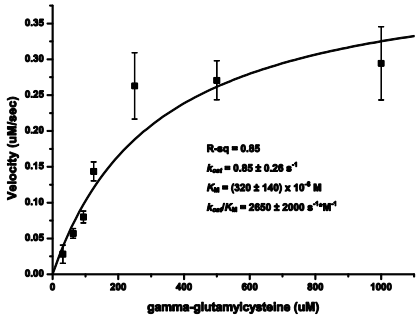

Figure S2h: phosphate buffer, pH 6.0 $EgtB_{Y377F}$, [TMH] = 400 µM

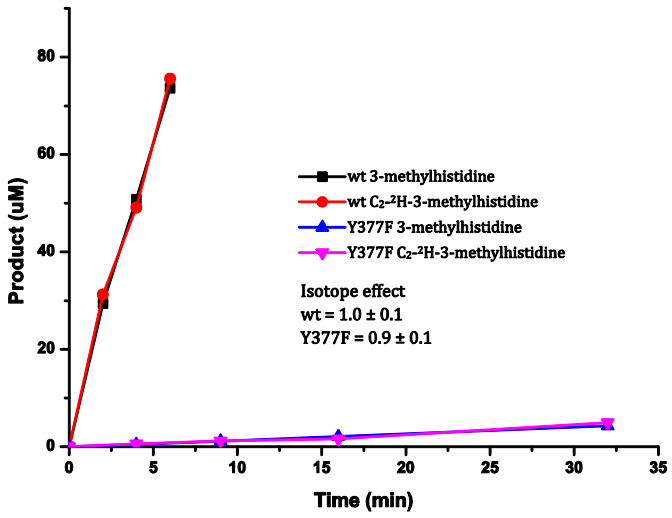

Figure S3. Left: Determination of the substrate KIE for $EgtB_{wt}$ and $EgtB_{Y377F}$. C_2-deuterated N-α-trimethyl histidine was prepared as follows: a 5 mM solution of N-α-trimethyl histidine in D_2O was acidified to pD 5.0 with 20 % DCl. This solution was incubated at 90 °C for 72 h in a sealed glass vial. Specific and complete deuteration of the imidazol C_2 position was confirmed by ESI-MS (m/z calc.: 199.13; meas. :199.1) and ^{1}H NMR (400 MHz, D_2O) δ ppm 7.26 (s, 1H), 3.88

(dd, J = 12.0, 3.9 Hz, 1H), 3.44 – 3.24 (m, 2H), 3.23 (s, 9H). Catalytic activity assayed in 250 µL reactions containing 100 mM HEPES buffer pH 8.0, 100 mM NaCl, 2 mM TCEP, 2 mM ascorbate, 4 µM $FeSO_4$, 400 µM TMH or C_2-deuterted TMH, 1.2 mM γGC and 1.2 µM EgtB or $EgtB_{Y377F}$. The reactions were incubated at 26°C; product formation was monitored by cation exchange HPLC using 20 mM phosphoric acid at pH 2 as a mobile phase.[4] Compounds were eluted in a NaCl gradient. All HPLC chromatograms were recorded at 265 nm.

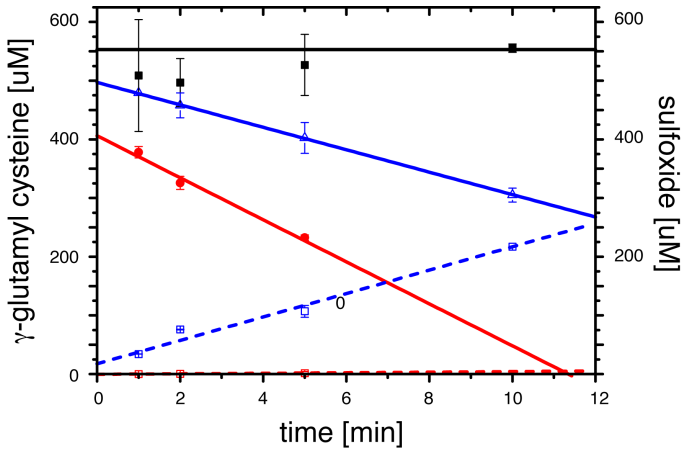

Figure 4. $EgtB_{wt}$ and $EgtB_{Y377F}$ catalyzed consumption of γGC and sulfoxide production in presence of 0.5 mM TMH, 0.55 mM γGC, 2 mM ascorbate, 100 mM HEPES buffer pH 8.0, and 0.9 uM of either enzyme. γGC consumption by $EgtB_{wt}$: 0.4 ± 0.02 s^{-1} (blue line); by $EgtB_{Y377F}$: 0.6 ± 0. 1 s^{-1} (red line). Sulfoxide production by $EgtB_{wt}$: 0.3 ± 0.06 s^{-1} (blue dash); by $EgtB_{Y377F}$: < 0.002 s^{-1} (red dash). In absence of enzyme γGC was not consumed (black line)

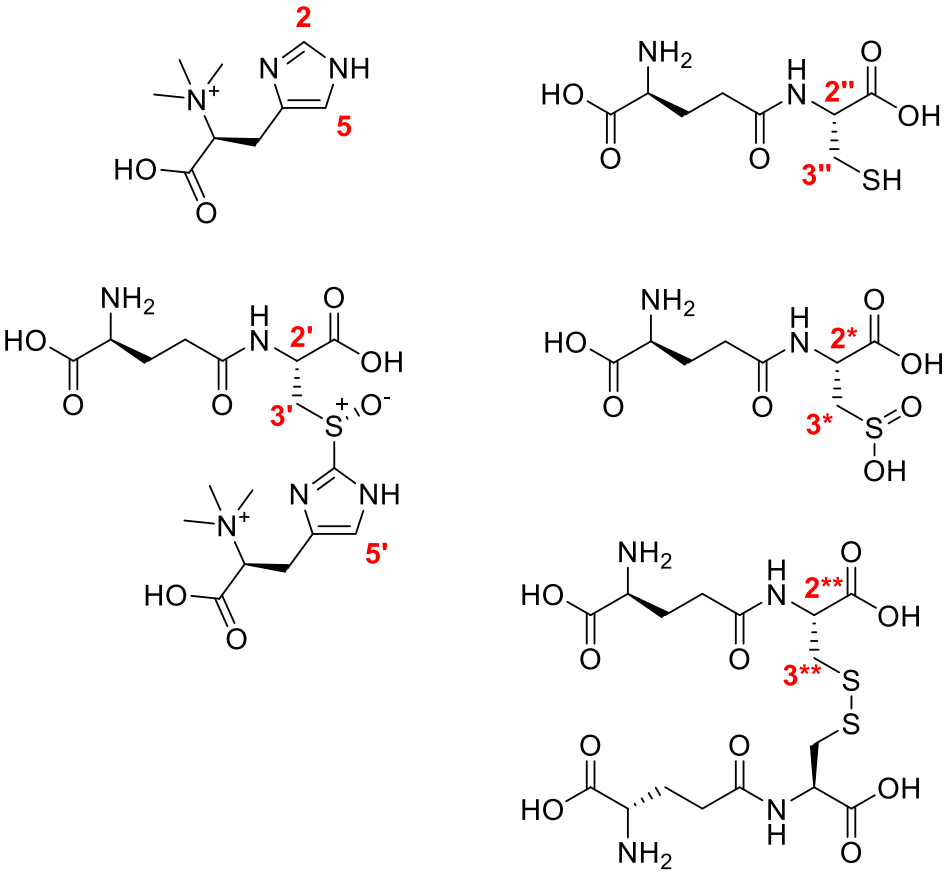

Figure S5. EgtB substrates and products were quantified by ^{1}H NMR based on α- and β-protons of the cysteine moiety and the aromatic protons of TMH.

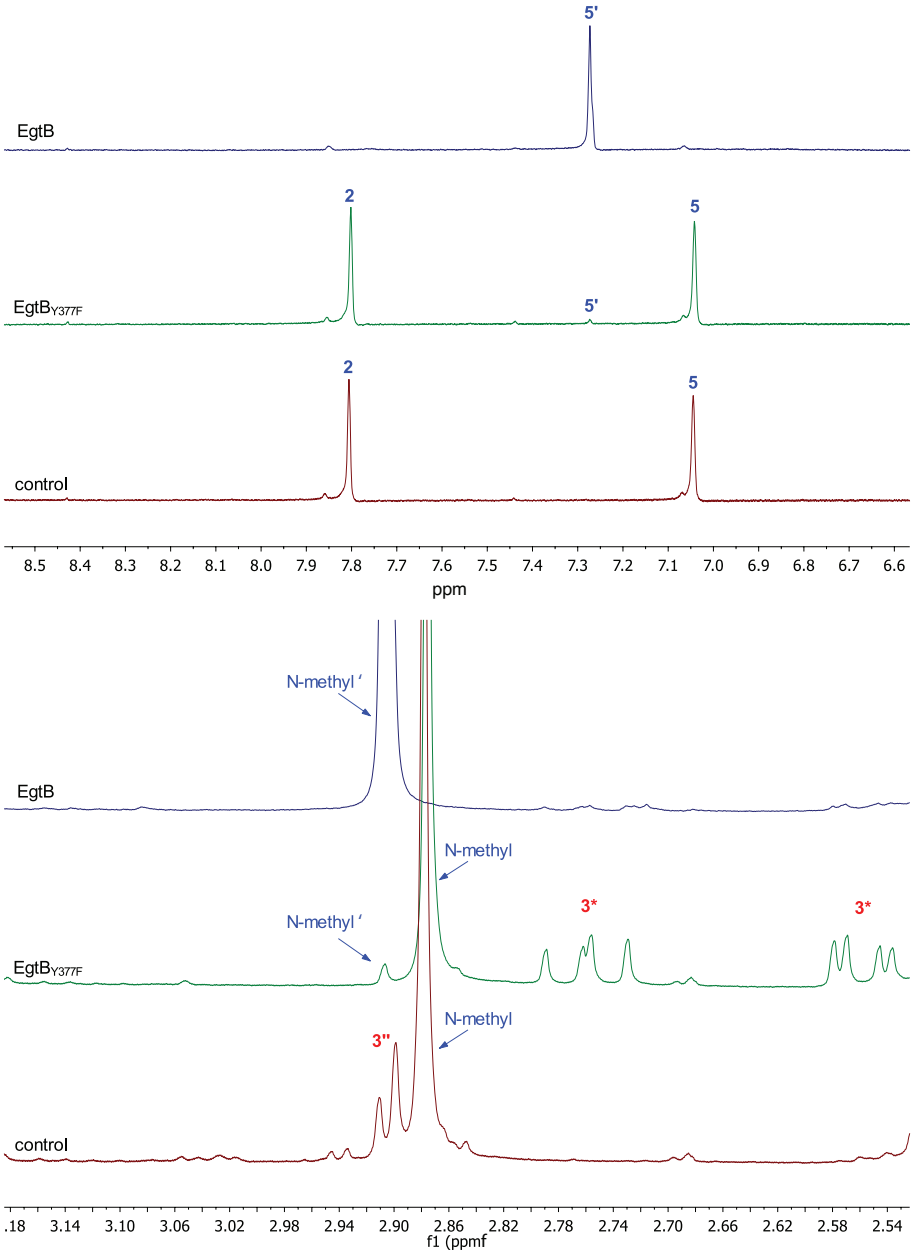

Figure S6. The product of $EgtB_{Y377F}$ catalyzed γGC consumption was identified as γGC dioxide by ^1H NMR. Reactions containing 100 mM sodium phosphate buffer pH 8.0, 100 mM NaCl, 2 mM ascorbate, 4 µM $FeSO_4$, 2 mM TCEP, 0.5 mM DMH, 0.5 mM γGC and 1 µM $EgtB_{wt}$ or $EgtB_{Y377F}$ were incubated for 12 h at 26°C. A reaction containing no enzyme was used as control experiment. The reactions were lyophilized, the residue was dissolved in D_2O. **Top: aromatic region:** ^1H NMR (400 MHz, D_2O): $EgtB_{wt}$ reaction: δ 7.27 (s, 1H, 5'); $EgtB_{Y377F}$ reaction: δ 7.80 (s, 1H, 2), 7.27 (s, 1H, 5'), 7.04 (s, 1H, 5); control reaction: δ 7.81 (s, 1H, 2), 7.04 (s, 1H, 5). **Bottom: aliphatic region:** $EgtB_{wt}$ reaction: δ 2.91 (s, 6H, N-methyl'); $EgtB_{Y377F}$ reaction: δ 2.91 (s, 6H, N-methyl'), 2.88 (s, 6H, N-methyl), 2.76 (dd, *J* = 13.1, 10.7 Hz, 1H, 3*), 2.56 (dd, *J* = 13.3, 3.7 Hz, 1H, 3*); control reaction: δ 2.95 – 2.83 (m, 8H, N-methyl, 3'', 3'').

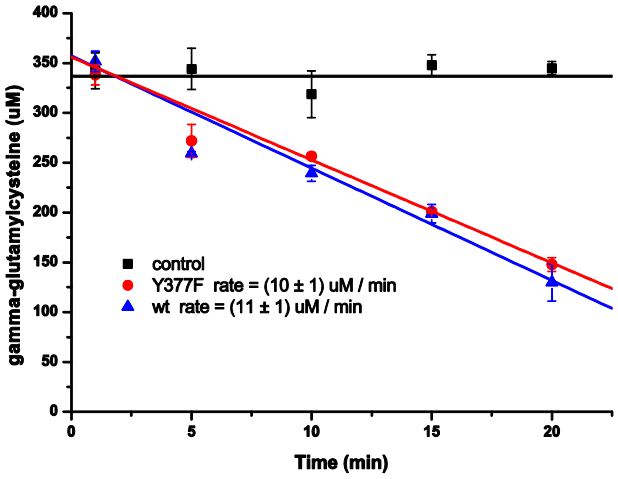

Figure S7. γGC consumption in absence of TMH. Reactions containing 100 mM HEPES buffer pH 8.0, 100 mM NaCl, 4 µM $FeSO_4$, 2 mM ascorbate, 0.35 mM γGC and 21 µM of either $EgtB_{wt}$ or $EgtB_{Y377F}$ were incubated at 26°C. Residual γGC was quantified a coumarin adduct by RP-HPLC.

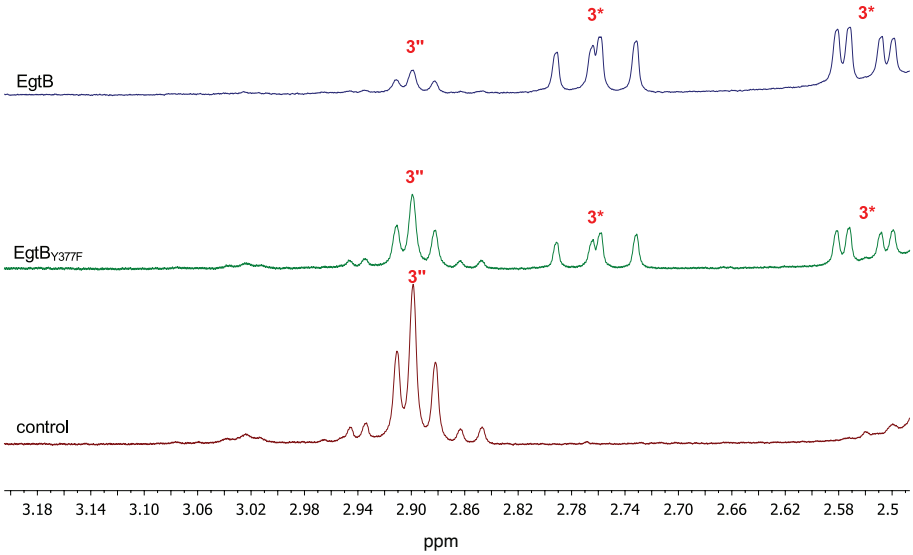

Figure S8. The product of $EgtB_{wt}$ and $EgtB_{Y377F}$ catalyzed TMH-independent γGC consumption was identified as γGC dioxide by ^1H NMR. Reactions containing 100 mM sodium phosphate buffer pH 8.0, 100 mM NaCl, 20 µM $FeSO_4$, 2 mM ascorbate, 2 mM TCEP, 0.5 mM γGC and 5 µM $EgtB_{wt}$ or $EgtB_{Y377F}$ were incubated for 12 h at 26°C. ^1H NMR (400 MHz, D_2O): $EgtB_{wt}$ reaction: δ 2.96 – 2.83 (m, 2H, 3''), 2.76 (dd, *J* = 13.3, 10.9 Hz, 1H, 3*), 2.56 (dd, *J* = 13.3, 3.7 Hz, 1H, 3*); $EgtB_{Y377F}$ reaction: δ 2.96 – 2.83 (m, 2H, 3''), 2.76 (dd, *J* = 13.2, 10.7 Hz, 1H, 3*), 2.56 (dd, *J* = 13.3, 3.7 Hz, 1H, 3*); control reaction: δ 2.96 – 2.83 (m, 2H, 3'').

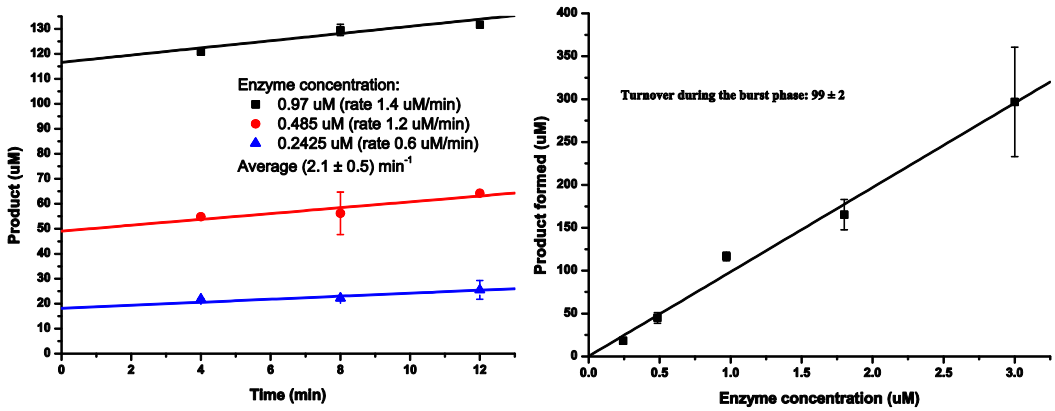

Figure S9. Rate of autoxidation by $EgtB_{wt}$. In absence of ascorbate $EgtB_{wt}$ loses activity within the first 1 - 2 min of catalysis owing to reversible autoxidation of EgtB to the inactive iron (III) form. In presence of ascorbate this inactive species does not accumulate because reduction to the active iron (II) form is rapid. Reduction by γGC is much slower which limits ascorbate-independent turnover in the steady state (starting after 4 min) to 2 min^{-1}. Linear regression of this slow phase to t = 0 gives an estimate of how many turnovers were completed before EgtB oxidizes the first time (**left**). These estimates from experiments with different enzyme concentrations plotted against enzyme concentrations reveal that each EgtB molecule catalyzes on average 100 turnovers before autoxiation occurs. In other words, k_{cat} is 100 fold faster than $k_{autoxidation}$ i.e. $k_{autoxidation} = k_{cat}/100$ (**right**). Catalytic rates were determined in reactions containing 100 mM HEPES pH 8.0, 100 mM NaCl, 4 eq. of $FeSO_4$, 2 mM TCEP, 0.4 mM TMH, 1.2 mM γGC, and 0.25-3 μM EgtB.

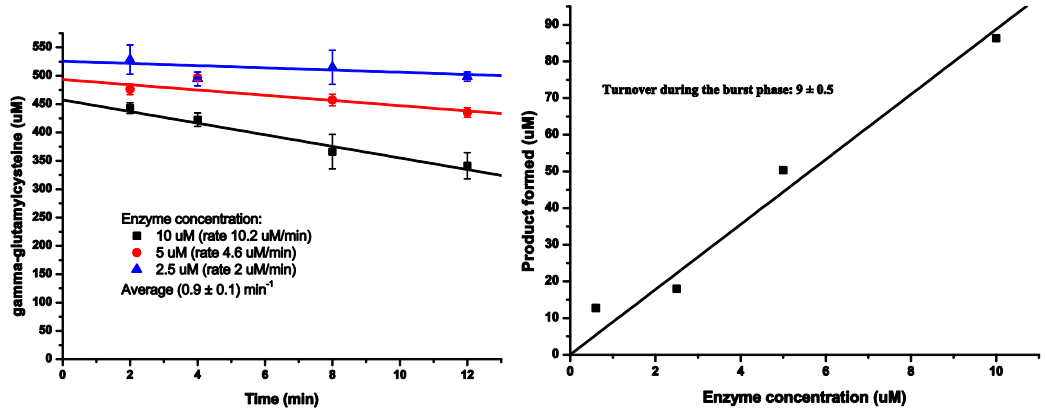

Figure S10. Rate of autoxidation by $EgtB_{Y377F}$. In absence of ascorbate $EgtB_{Y377F}$ also loses activity within the first 1 - 2 min due to reversible autoxidation. Determination of $k_{autoxidation} = k_{cat}/9$ was achieved using the same methodology as described above. Catalytic rates were determined in reactions containing 100 mM sodium phosphate buffer pH 8.0, 100 mM NaCl, 4 eq. of $FeSO_4$, 2 mM TCEP, 0.4 mM TMH, 0.55 mM γGC, 2.5-10 μM $EgtB_{Y377F}$.

Solvent KIE. To measure the solvent KIE standard reaction mixture containing 100 mM HEPES, 100 mM NaCl, 2 mM TCEP, 4 μM $FeSO_4$, 2 mM ascorbate, 1 mM DMH was adjusted to pH 8.0 or 7.6 (final pD = 8.0).[5] Premixtures were lyophilized and then dissolved in H_2O or D_2O. The reactions were initiated by addition of enzyme. The final deuterium to hydrogen ratio was estimated to be at least 8.5:1.

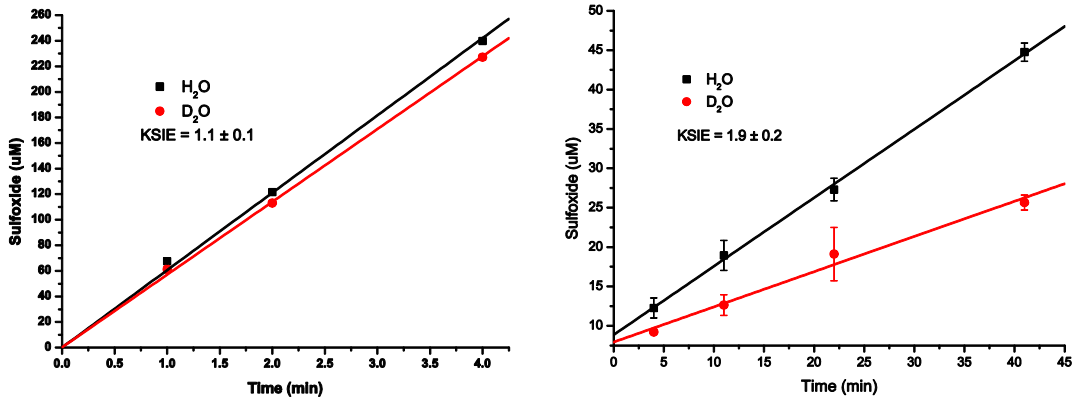

Figure S11. Solvent KIEs on $EgtB_{wt}$ (**left**) and $EgtB_{Y377F}$ (**right**) catalyzed sulfoxide production were determined at 26°C in reactions containing 100 mM HEPES, 100 mM NaCl, 2 mM TCEP, 2 mM ascorbate, 4 or 40 μM $FeSO_4$, 1 mM TMH and 1.2 mM γGC, 1.4 μM $EgtB_{wt}$ or 10 μM $EgtB_{Y377F}$.

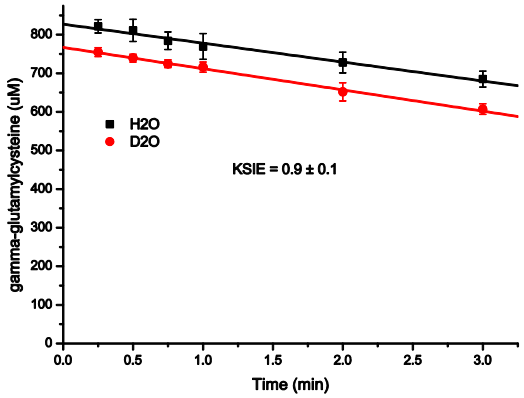

Figure S12. The solvent KIE on $EgtB_{Y377F}$ catalyzed γGC dioxygenation was determined at 26°C in reactions containing 100 mM HEPES, 100 mM NaCl, 2 mM TCEP, 4 μM $FeSO_4$, 2 mM ascorbate, 1 mM DMH, 0.8 mM γGC, and 0.5 μM $EgtB_{Y377F}$. Consumption of γGC was monitored as described above.

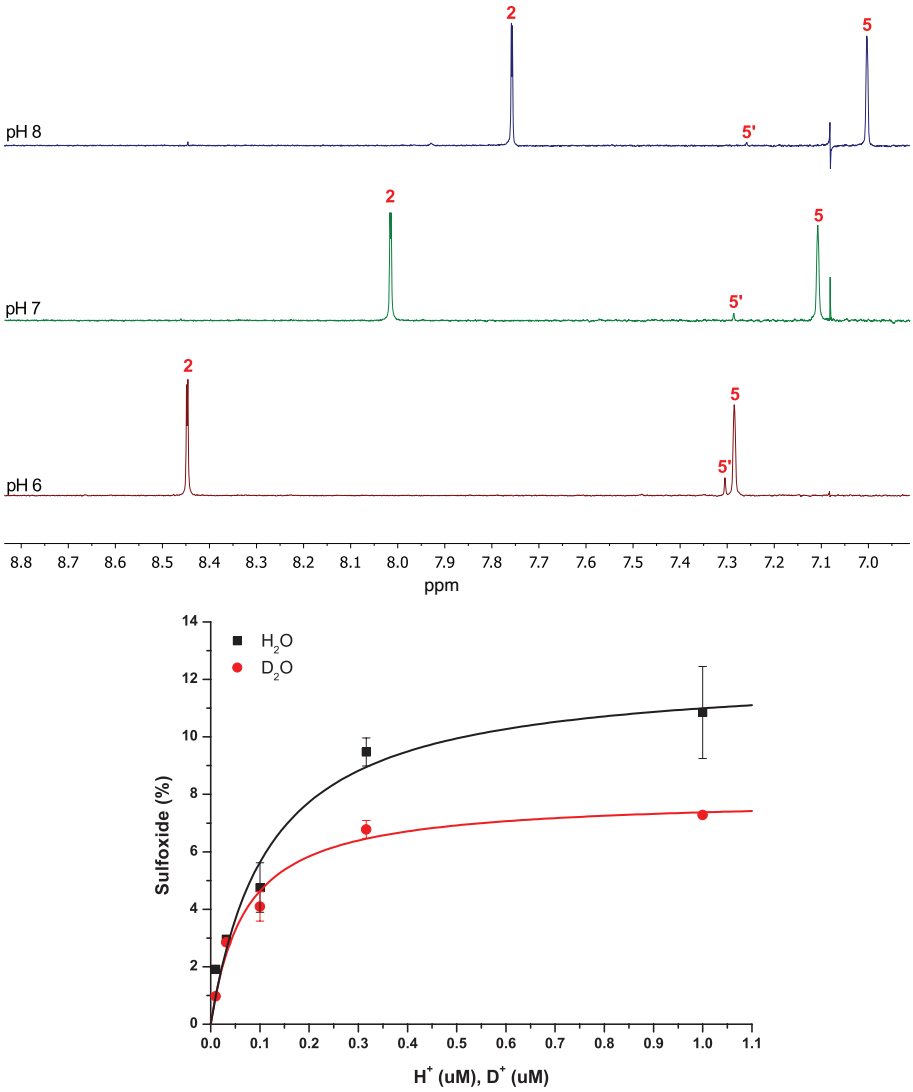

Figure S13. To determine the dependence of $EgtB_{Y377F}$ catalyzed sulfoxide production on buffer pH we determined the ratio between substrate TMH and product sulfoxide in completed reactions by ^1H NMR. The same analysis was repeated in D_2O, to probe the pH dependence of the solvent KIE. The reactions (in H_2O or D_2O) contained 100 mM sodium phosphate buffer at pH (or pD) 8.0, 7.0 or 6.0, 100 mM NaCl, 2 mM ascorbate, 4 μM $FeSO_4$, 2 mM TCEP, 0.5 mM TMH, 0.75 mM γGC and 1 μM $EgtB_{Y377F}$. The reactions were incubated at 26°C. After 12 h the solutions were lyophilized and dissolved D_2O for NMR analysis (**top**). The aromatic signals of TMH (C_2-H and C_5-H) and of sulfoxide ($C_{5'}$-H) were used to compute the percentage of consumed TMH, i.e. produced sulfoxide. These values (obtained at pH 6.0, 6.5, 7.0, 7.5 and 8.0) were plotted against proton concentration and fitted with a hyperbolic function of the form:

$$[\text{sulfoxide}] = [\text{sulfoxide}]_{max}[H^+]/(K_{M,proton} + [H^+])$$

This analysis revealed that a) the solvent KIE remains > 1.5 in the examined pH range; b) increased proton concentration stimulates sulfoxide production by $EgtB_{Y377F}$; c) pH dependent acceleration saturates with a pH below 7 (**bottom**).

References

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