Supplemental Figures:

Fig. S1

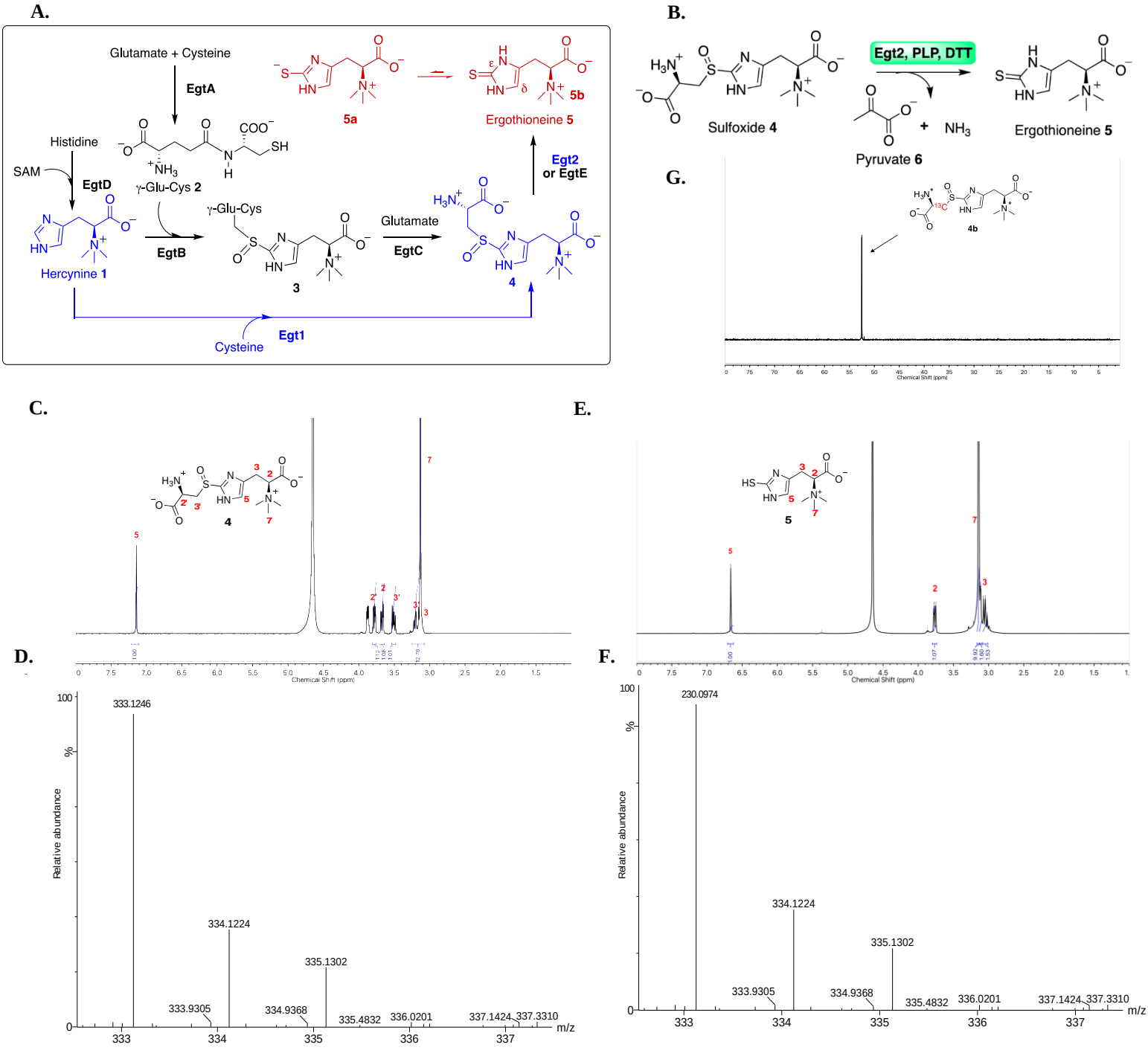


Fig. S1. Biosynthetic pathways of ergothioneine and Chemical Characterization and validation of Egt2, Related to Figure 1. (**A**). Two distinct biosynthetic pathways of ergothioneine production. The mycobacterium *M. smegmatis* pathway involves EgtA-EgtE enzymes (shown in black) while the fungal *N. crassa* route involves Egt1-Egt2 enzymes (shown in blue).(**B**) . Reaction catalyzed by Egt2 from fungal *N. crassa*. **(C-D)** Characterization of sulfoxide substrate **4** prepared through enzymatic reaction of Egt1 from *N. crassa*. (**C**) ^{1}H NMR spectrum of the sulfoxide substrate **4**: δ 3.10-3.19 (m, 13H), δ 3.48 (dd, J = 3.9, 15.1 Hz, 1H), δ 3.65 (dd, J = 3.9, 8.8 Hz, 1H), δ 3.77 (dd, J = 3.9, 11.9, 1H), δ 7.12 (s, 1H). (**D**) Molecular ion region in the HRMS spectrum of the sulfoxide substrate **4**. The calculated [M-H]$^{-}$ in negative mode for compound was 333.1233, and found 333.1246. (**E-F**) Characterization of ergothioneine **5**. **(E)** ^{1}H NMR spectrum of ergothioneine **5** from Egt2 reaction with DTT as reductant: δ 3.05 (t, J = 12.5 Hz, 1H), δ 3.12 (dd, J = 3.4, 12.5 Hz, 1H), δ 3.14 (s, 9H), δ 3.75 (dd, J = 3.6, 10.2 Hz, 1H), δ 6.66 (s, 1 H). **(F)** Molecular ion region of the HRMS spectrum of ergothioneine **5** from Egt2 reaction. The calculated [M-H]$^{-}$ in negative mode for compound was 230.0963, and found 230.0974. (**G**) ^{13}C NMR spectrum of [^{13}C]-labeled sulfoxide substrate **4b** from *N. crassa* Egt1. (125 MHz, D_2O) of [^{13}C]-labeled sulfoxide substrate **4b**: δ 52.52.

Fig. S2

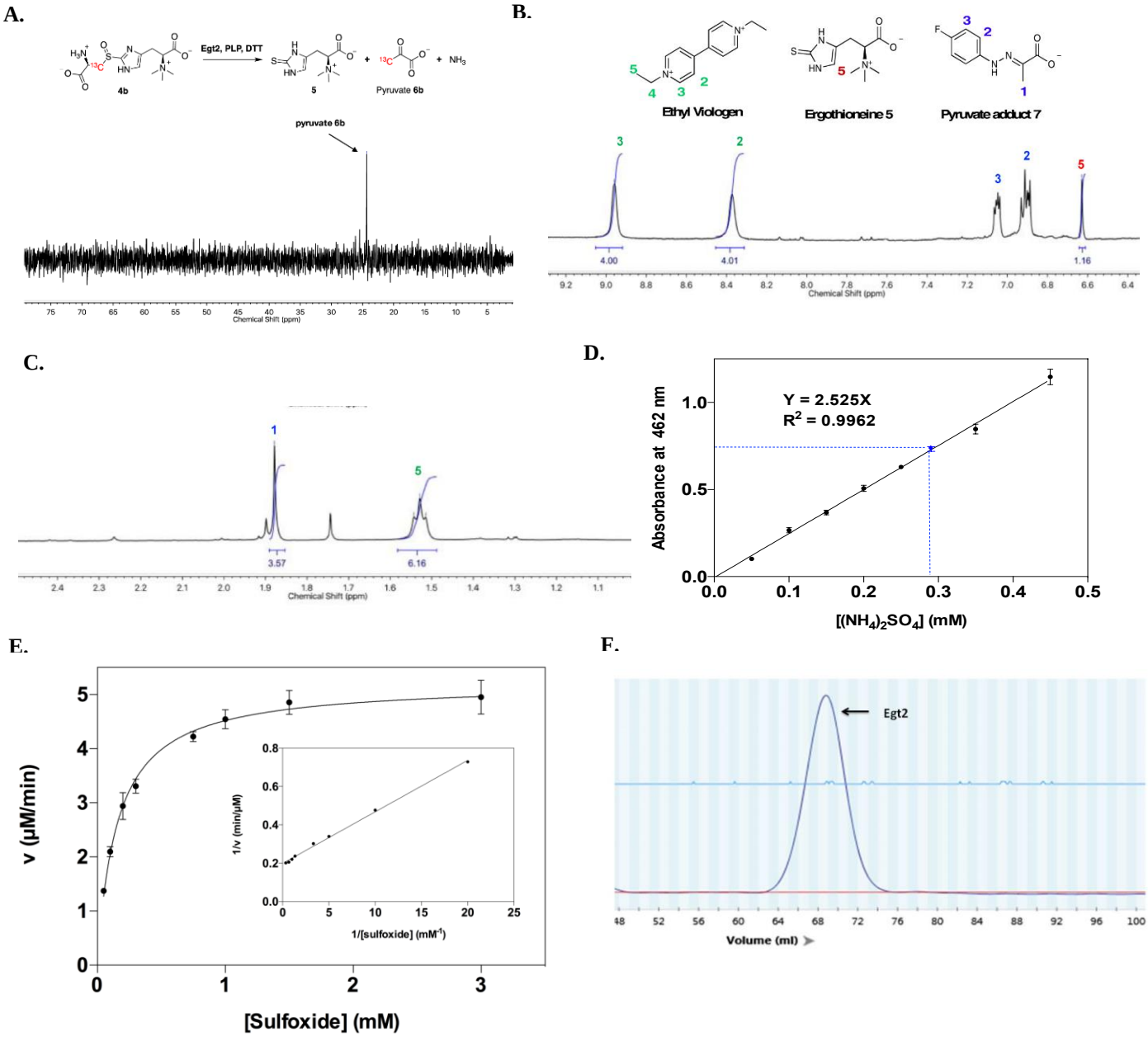


Fig. S2. Characterization of Egt2-mediated C-S cleavage, Related to Figure 1 and Figure 2. (A) ^{13}C NMR spectrum of Egt2 reaction using **4b** as the substrate showing the presence of [3-^{13}C]-pyruvate **6b** as the product in addition to ergothioneine and NH_4^+ formation. (125 MHz, D_2O) of [3-^{13}C]-labeled pyruvate **6b**: δ 24.35. (**B-C**) Quantifying the ratio between pyruvate and ergothioneine produced in Egt2 reaction using ^1H NMR spectrum of: **(B)** expanded regions between 6.4 ppm to 9.2 ppm; **(C)** expanded regions between 1.0 ppm to 2.5 ppm. (**D**) NH_4^+ titration standard curve. The standard solution was prepared using $(NH_4)_2SO_4$. The A_{462} of Egt2 reaction sample was 0.7333 ± 0.06 (shown in blue). Error bar represent SD. The amount of NH_4^+ produced from Egt2 reaction was determined to be ~ 1:1. (**E**) Egt2 wild-type steady-state kinetic analysis at 25°C. A 1-mL assay contained 0.13 mM NADH, 1 mM DTT, 22.5 U/mL LDH (2000× of Egt2 activity in the assay) in a universal buffer (25 mM NaOAc, 25 mM MES, 25 mM glycine, and 75 mM Tris) pH 8.0 and various amount of sulfoxide substrate **4**, and 10 nM Egt2 enzyme. The coupled assay was monitored at 340 nm using a Varian Cary Bio 100 spectrophotometer and the data were fitted by GraphPad Prism. Wild-type Egt2 exhibited a Michaelis constant (K_m) for the sulfoxide substrate of 155.2 ± 9.6 μM and k_{cat} of 8.7 ± 0.1 s^{-1}. Error bar represent SD. (**F**) The gel filtration profile of Egt2 (theoretical molecular weight of 56 kDa for Egt2 monomer) shows an elution volume of 71 ml corresponding to a molecular weight of 114 kDa in solution. The molecular weight was calculated from the gel filtration calibration curve y(ml) = -27.588 log(mw(Da))+210.54 .

Fig. S3

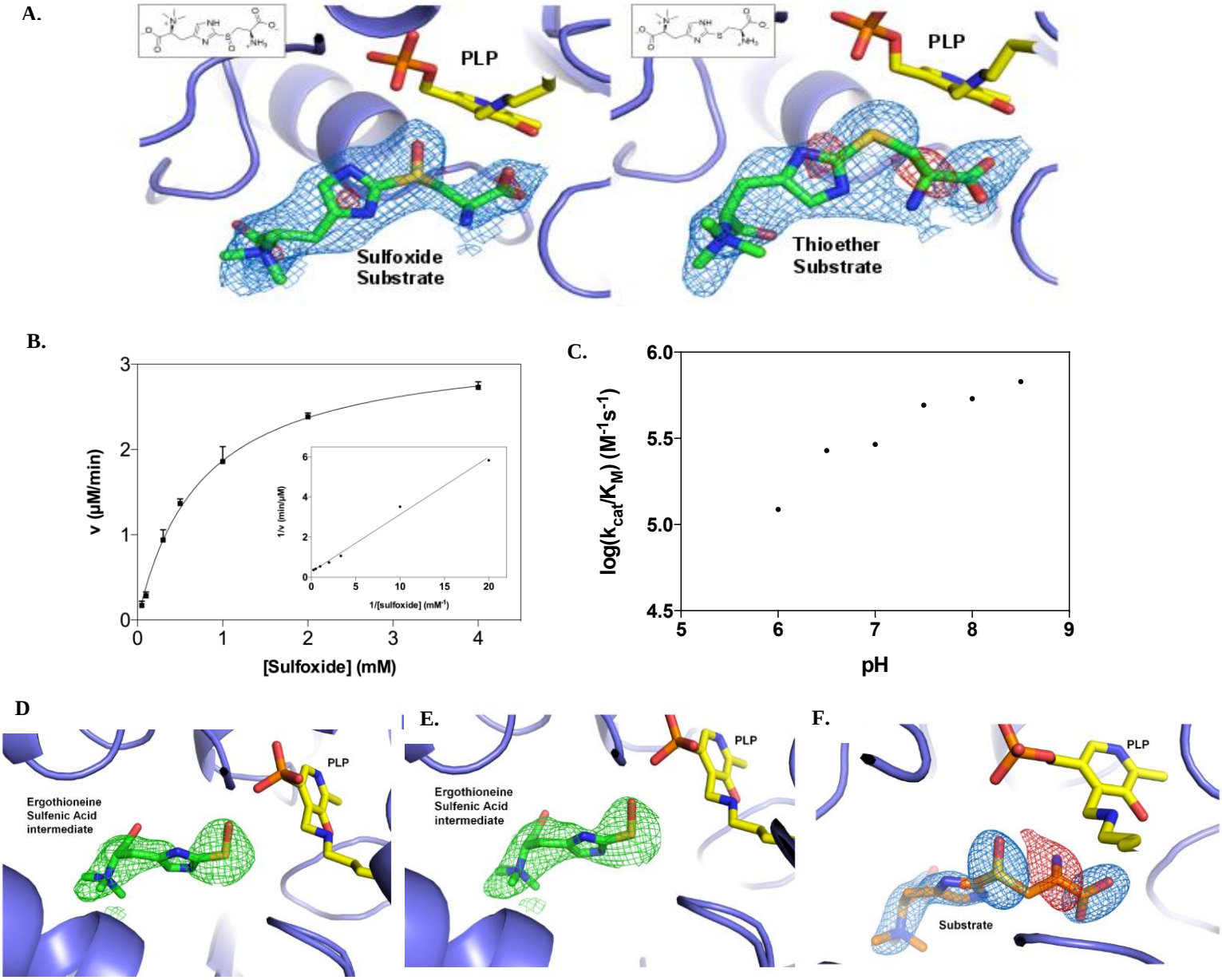
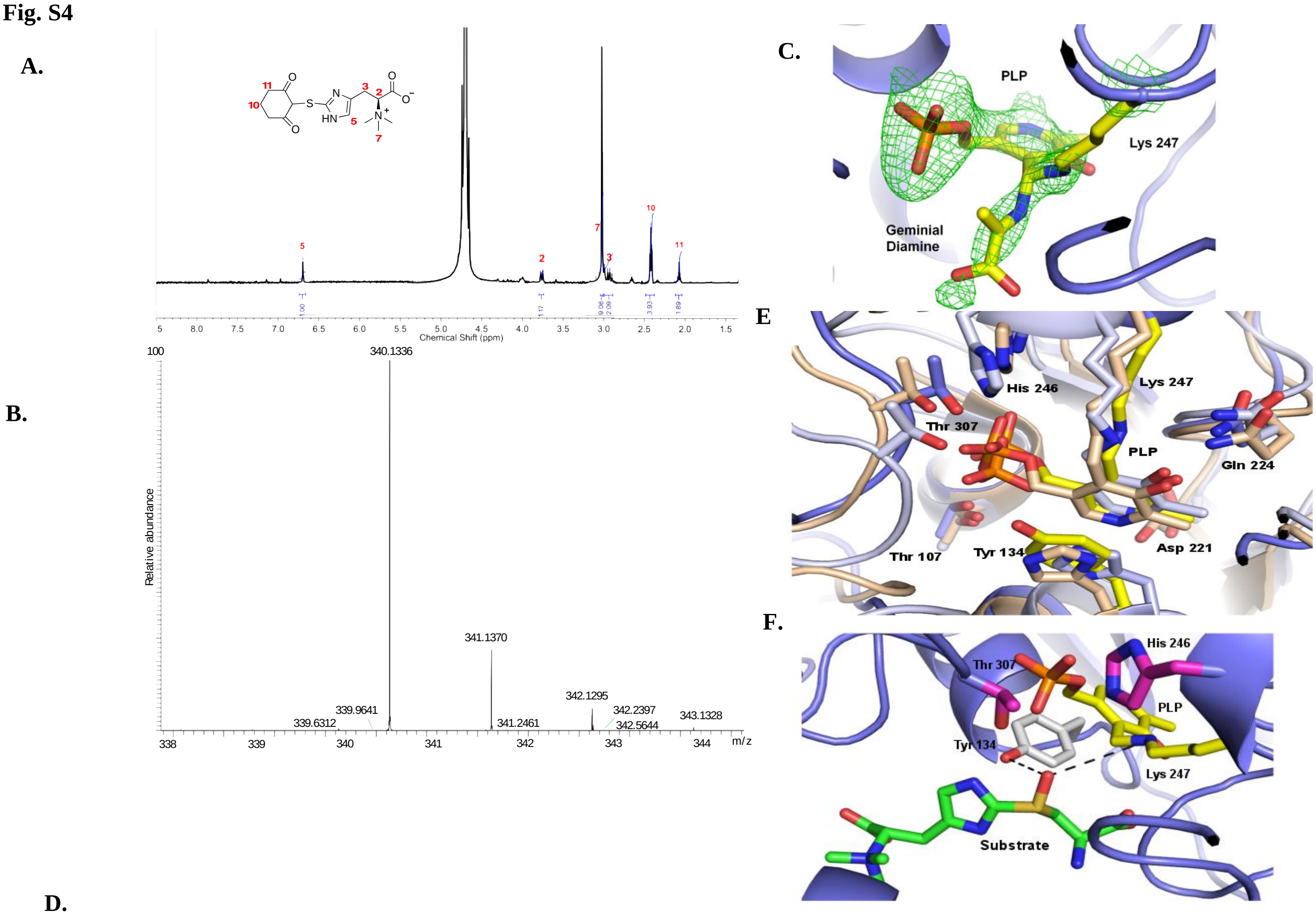


Fig. S3. Y134 mutation in Egt2, Related to Fig. S3.(**A**) The electron density for the sulfoxide substrate and thioether substrate at 2mFo-DFc contour of 0.8σ and mFo-DFc 3.2σ. **(B)** Egt2 Y134F steady-state kinetic analysis at 25°C. A 1-mL assay contained 0.13 mM NADH, 1 mM DTT, 22.5 U/mL LDH (2000× of Egt2 activity in the assay) in a universal buffer (25 mM NaOAc, 25 mM MES, 25 mM glycine, and 75 mM Tris) pH 7.0 and various amount of sulfoxide substrate **4**, and 12 nM Egt2Y134F enzyme. The coupled assay was monitored at 340 nm using a Varian Cary Bio 100 spectrophotometer and the data were fitted by GraphPad Prism. Egt2 exhibited a K_m of 743.2 ± 52.5 μM and k_{cat} of 3.6 ± 0.1 s^{-1}. Error bar represent SD. (**C**) log(k_{cat}/K_m) under varying pH for the Egt2Y134F. Kinetic data higher than pH 8.5 was not shown since protein lost all activity due to unfolding. (**D**)The mFo-DFc omit map of the active site of Egt2 Y134F (contoured to 2.3σ) after bound substrate was transferred to a high pH environment. The ergothioneine sulfenic acid intermediate was shown built into the density to facilitate visualization but was not included in the omit map calculation. **(E)** The mFo-DFc Polder OMIT map at 4.5σ for the ergothioneine sulfenic acid intermediate generated using the phenix.polder. (**F**) The mFo-DFc and 2mFo-DFc maps calculated with the substrate hercynylcysteine sulfoxide modeled in the density. The 2mFo-DFc is contoured to at 1σ and mFo-Fc at 3σ with negative density observed at C-S bond.

Fig. S4



D.

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                                              115                                                                181
gi|164424097|ref|XP_001728131.1|     RNIVWSADGKDEILYFDTIYGACGKTIDYVIEDK-RGIVSSRCIPLIYPAEDDDVVAAFRDAIKKSR
gi|725981043|gb|KHE84184.1|          RNIVWSADGKDEILYFDTIYGACGKTIDYVIEDK-RGIVSSRCIPLIYPAEDDDVVAAFRDAIKKSR
gi|1003740426|gb|KXX76625.1|         RNLVWNPDGKDEILYFDTIYNGCARTIDYVIEDR-QGLVSSRCIDLSYPCEDDAVVAAFKSGVKASL
gi|1020431150|gb|KZL67276.1|         RNLTWAPDGLDEILYFSTIYGGCAKTIDYIVDTR-SGLVSSRSIPLICPSEDDEIVALFRDTVTKSR
gi|576045780|ref|XP_006696763.1|     RSLPWSQDKTDDILYFETIYGGCAKTVDYVVEDR-RGLITSRCIPIQYPCSDAVLVEAFRSAVAASR
gi|1020431505|gb|KZL67612.1|         RNLVWAPDGLDEILYFSTIYGGCAKTIDYIVDTR-SGLVSSRGIPLSYPLEDDEIVAIFRDTVTKSR
gi|1000220050|gb|KXJ89061.1|         RNLNWNPDGKDEILYFSTIYGACGKTIDYIVDSHPPGTVSSRCITATYPAEDTELLAAFHAAVKTST
gi|1039274734|gb|OBR13003.1|         RNLIWAPDGLDEILYFSTVYGGCAKTIDYIVDTR-LGLVSSRSIPLTYPLEDDEVVALFRDAVAQSH
gi|347829610|emb|CCD45307.1|         RNLAWNEDGKDEVLYFSTIYGACGLTIRYVSEAS-RGLVQPRQITFQYPIEDDELVGLFEKAIEASK
gi|927757853|gb|KPA41089.1|          RNLKWNEDGKDVIISFSTIYEACGKVADYLVDYYN-ENVAHREVELEYPIDDDEIIKRFEDTVKKIE
gi|814600295|gb|KKF94950.1|          RNLEWDSDCKDEILYFETIYGACGKTIDFVVDFS-YGRASSRGIPIRYPCETEEIVACFQEAVKASR
gi|517317783|emb|CCT68964.1|         RNMKWNEDGKDVIISFSTIYEACGKVADYYVDYYN-EKVTHREIELTYPLDDDEIIKKFEDTVKKIE
gi|477524462|gb|ENH76403.1|          RNLTWDADGRDEILYFNTIYGACGKTISYIVDTR-QGLVSARGITLTYPLEDEEIIAVFRSTVAGSR
gi|949390134|dbj|GAP86944.1|         HNLTWDDDGKDEIVYFSTVYSGCGKIIDHEVDTG-LGRVSSRAISITYPCEDEDIVKAFHDALDSIK
gi|1032868490|gb|OAQ75242.1|         KNLAWNDDAKDVILTFSTAYEACAKVADYIVDSTG-GKVEHREITIDYPLEDEEILQAFREAVKAVE
gi|821075074|gb|KKY29339.1|          RSLPWSEDGKDEIIHFSTIYGGIGKTVDYVVDST-YGRVSSRSIELAYPISDDAIIAKFRQAVQESR
gi|908386352|gb|KND87102.1|          RNLVWADDDKDVVLTFSTVYEACGKVADYLVDYYG-GKVEHREIPIVYPLEDEDILQAFREAVRAIE
gi|589113425|ref|XP_006968735.1|     RNIKWDADGKDVALWFSTVYEACGKAIDFLYDYHGDGRLSSREIEIAYPIEDDEILRRFRSAVEQVR
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Fig. S4. Characterization of sulfenic acid-1,3-cyclohexanedione adduct 8 from Egt2 reaction in the presence of 1,3-cyclohexanedione trapping reagent, Related to Figure 4. (A) ^{1}H NMR spectrum: δ 1.83 (dt, J = 6.3, 12.8, 2H), δ 2.35 (t, J = 6.3, 4H), δ 2.98 (dd, J = 6.3, 12.8, 2H), δ 3.02 (dd, J = 3.9, 11.6 Hz, 1H), δ 3.09 (s, 9H), δ 3.73 (dd, J = 4.4, 10.8 Hz, 1H), δ 3.74 (dd, J = 3.9, 11.7 Hz, 1H), δ 6.68 (s, 1 H) **(B)** Molecular ion region of the HRMS spectrum of the adduct **8**. Calculated value for the adduct compound **8** $[M+H]^+$ (positive mode) was *m/z* 340.1326, and found *m/z* 340.1336. (**C**) The mFo-DFc omit map contoured at 2.5σ for the geminial diamine intermediate. (**D**) Sequence alignment of seventeen Egt2 homologs using ClustalOmega. Numbering is based on Egt2 sequence, which is the first sequence (shown in bold). The conserved Y134 is highlighted in yellow. **(E)** Superimposition of the wild-type Egt2 structure with structures of cysteine C-S lyase 1ELQ and cysteine desulfurase 4Q76. The PLP cofactor for all the three structures is shown as sticks; the one corresponding to Egt2 is shown in yellow, the structurally conserved residues interacting with the PLP have been shown as sticks. Labeling of the residues is according to the Egt2 sequence. The Y134 in wild-type Egt2 is shown in yellow sticks. (F) Interactions between Egt2 Y134F mutant and its substrate 4. The tyrosine at position 134 was modeled in by superimposing Egt2 Y134F mutant with the wild-type Egt2 and is shown as grey sticks. Residues H246 and T307 around K247 are shown as pink sticks. Hydrogen bonds between residues are shown by black dash lines.

Fig. S5

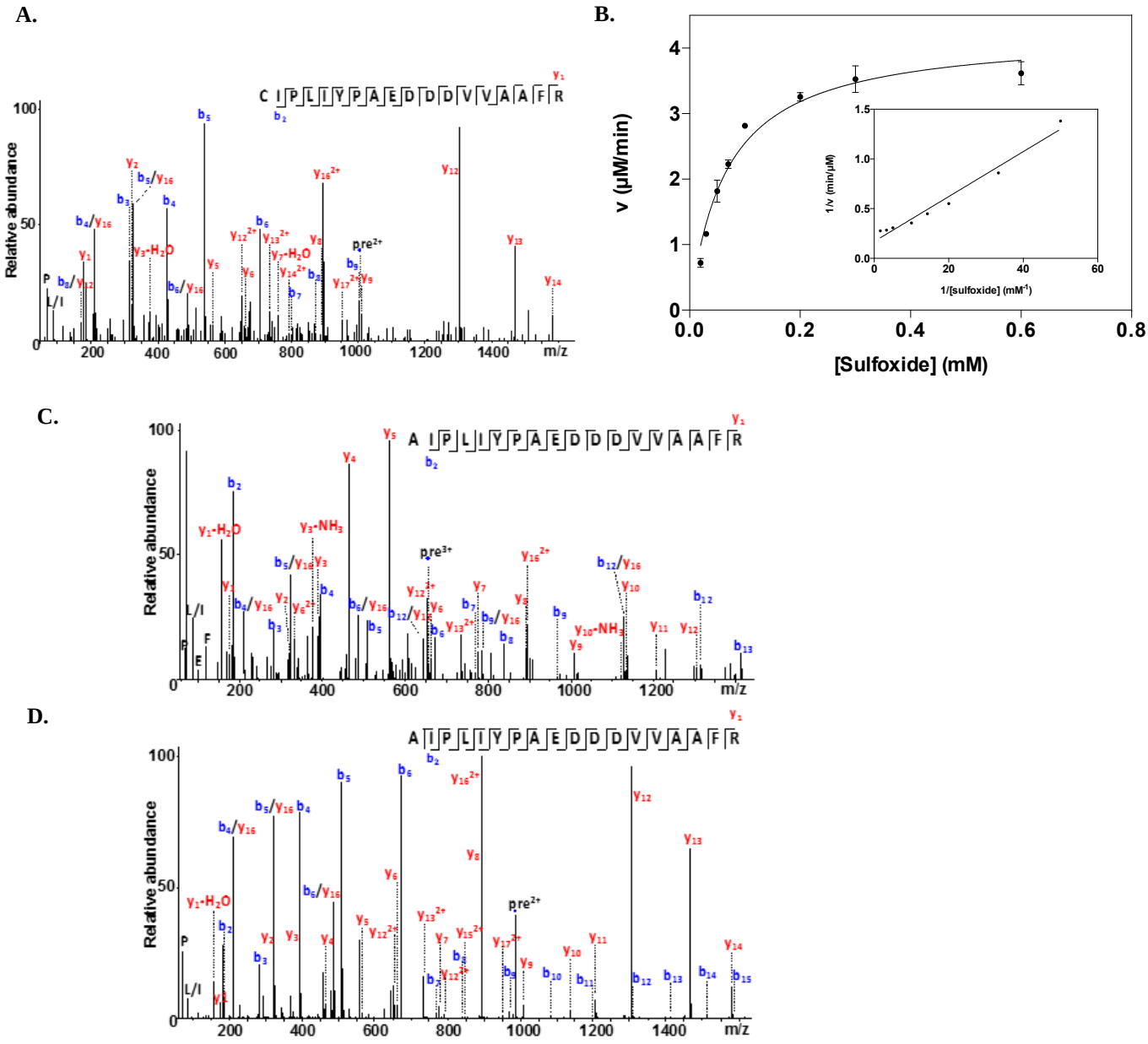
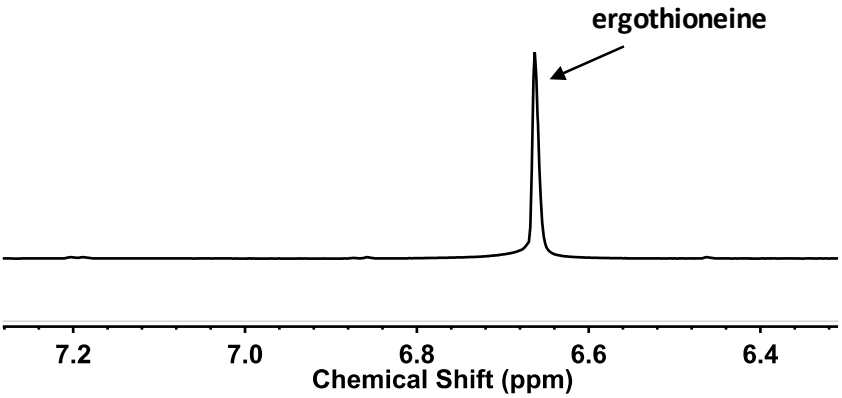


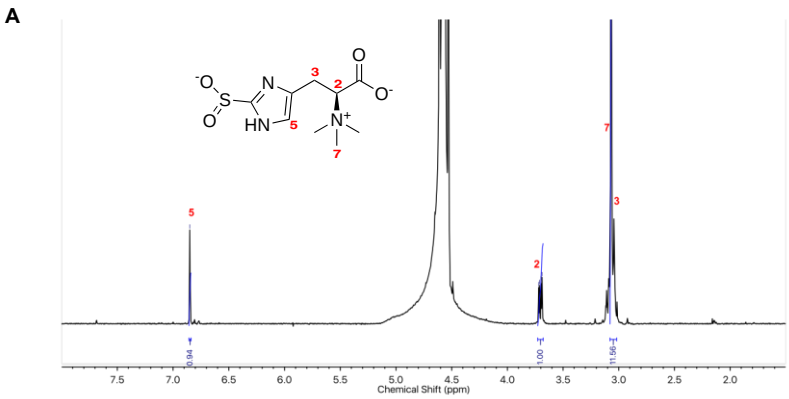
Fig. S5. MS/MS analysis of Egt2 wild-type and C156A from single-turnover reactions, Related to Figure 5. (**A**) MS/MS spectrum of a tryptic peptide (residue 156-173, doubly-charged parent ion at *m/z* 1003.9993) from wild-type Egt2. In this reaction, excess DTT was present. From this spectrum, it shows that there is no modification on this peptide. (**B**) Egt2 C156A steady-state kinetic analysis at 25°C. A 1-mL assay contained 0.13 mM NADH, 1 mM DTT, 22.5 U/mL LDH (2000× of Egt2 activity in the assay) in a universal buffer (25 mM NaOAc, 25 mM MES, 25 mM glycine, and 75 mM Tris) pH 8.0 and various amount of sulfoxide substrate **4**, and 10 nM Egt2 C156A enzyme. The coupled assay was monitored at 340 nm using a Varian Cary Bio 100 spectrophotometer and the data were fitted by GraphPad Prism. Egt2 C156A exhibited a K_m of 66.2 ± 5.8 μM and k_{cat} of 7.1 ± 0.2 s^{-1}. Error bar represent SD. (**C**) MS/MS spectrum of a tryptic peptide from Egt2 C156A before the single-turnover reaction (residue 156-173, triply-charged parent ion at *m/z* 659.0121). Modification was not observed on this peptide. (**D**) MS/MS analysis of a tryptic peptide from Egt2 C156A after the single-turnover reaction (residue 156-173, the doubly-charged parent ion at *m/z* 988.0218). There is no modification on this peptide.

Fig. S6

A.



B.



C.

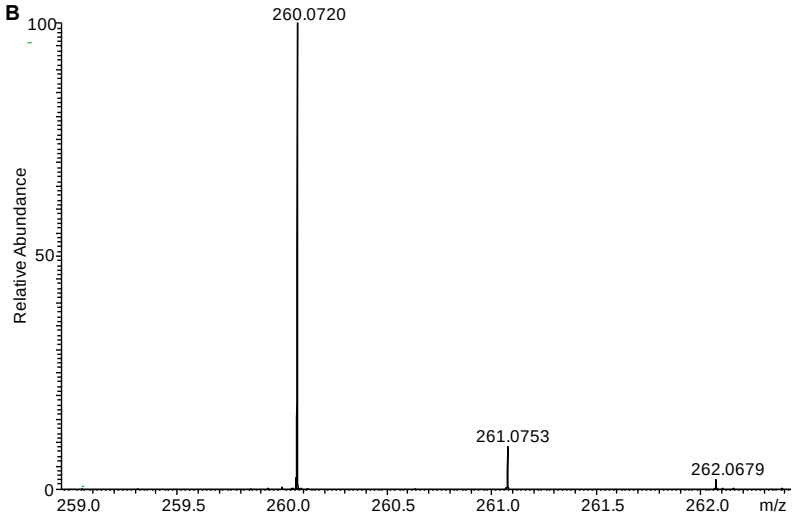


Fig. S6. **The Egt2 ET reaction in presence or absence of reductant. Related to Figure 5**. ^{1}H NMR analysis for (**A**) Egt2 C156A reaction in the presence of DTT showing ergothioneine as the product from this reaction.
(**B-C**) Characterization of **9** isolated from Egt2 reaction in the absence of reductant. **(B)**: ^{1}H NMR spectrum of ergothioneine-2-sulfinic acid **9**: δ 2.99-3.16 (m, 11H), δ 3.73 (dd, J = 3.8, 11.7 Hz, 1H), δ 6.85 (s, 1 H). **(C)**: Molecular ion region of the HRMS spectrum of ergothineine-2-sulfinic acid **9**. Calculated value for compound **9** as $[M-H]^-$ (negative mode) form was *m/z* 260.0711, and found *m/z* 260.0720.

Supplemental Table S1

Data collection for experimental phasing, Related to Figure 2.

Anomalous Data Collection	Egt2 + Platinum Soaking MAD dataset			Egt2+SelenoMet SAD dataset
Data collection	Peak	Inflection	Remote	
Space group	P 1 2_1 1	P 1 2_1 1	P 1 2_1 1	P 1 2_1 1
Cell dimensions				
a, *b*, *c* (Å)	103.4, 194.9, 108.2	103.9,195.7,108.8	103.9, 195.7,108.9	103.8,195.3,107.7
α, β, γ (°)	90.0, 91.2, 90.0	90.0, 91.4, 90.0	90.0, 91.4, 90.0	90.0, 91.3, 90.0
Resolution (Å)	50.00 - 3.50 (3.56 - 3.50) *	50.00-3.5 (3.56 - 3.50)	50.00 - 3.5 (3.56 - 3.5)	50.00 - 2.8 (2.85 - 2.80)
R_{sym}	0.117 (0.219)	0.130 (0.241)	0.090 (0.145)	0.106 (0.300)
$I/\sigma I$	10.86(4.94)	11.23 (4.63)	12.76 (6.00)	12.21 (3.11)
Completeness (%)	99.6(99.6)	99.90 (99.90)	99.6 (99.8)	99.7 (98.3)
Redundancy	3.4 (3.4)	3.9 (3.9)	3.7 (3.7)	3.7 (3.5)
Wavelength	1.07209	1.07236	1.04267	0.97648

*Highest resolution shell is shown in parenthesis.

Data collection and refinement statistics

	Egt2	Egt2 Y134F + Substrate pH 7.0	Egt2 Y134F + Substrate pH 8.0
Data collection	SetMet		
Space group	P 1 2_1 1	P 1 2_1 1	P 1 2_1 1
Cell dimensions			
a, *b*, *c* (Å)	103.8, 195.3, 107.8	103.9,195.1,107.6	104.0, 195.2,110.1
α, β, γ (°)	90.0, 91.3, 90.0	90.0, 91.3, 90.0	90.0, 91.5, 90.0
Resolution (Å)	50.00 - 2.30 (2.34 - 2.30)	50 - 2.57 (2.61 - 2.57)	50 - 2.45 (2.49 - 2.45)
R_{sym}	0.129 (0.529)	0.140 (0.630)	0.140 (0.763)
$I/\sigma I$	11.45 (1.79)	10.69 (1.50)	8.46 (1.41)
Completeness (%)	91.00(61.20)	99.4 (95.3)	91.2 (81.8)
Redundancy	5.9 (5.0)	3.1 (2.9)	2.9 (2.8)
Wavelength	0.97648	1.03320	1.03320
Refinement			
Resolution (Å)	49.36 - 2.30 (2.37 - 2.30)	48.82 - 2.56 (2.62 - 2.56)	49.58 - 2.45 (2.51 - 2.45)
No. reflections	154894	135254	146021
R_{work}/ R_{free}	0.2101/0.2410	0.2225/0.2793	0.2220/0.2717
No. atoms			
Protein	27984	27978	27896
Ligand/ion	21	44/15	32/24
Water	992	299	412
B-factors (Å^2)			
Protein	32.0	51.3	41.6
Ligand	-	62.8	66.1
Ligand(formate ion)	32.3	47.2	41.9
Water	29.3	44.2	38.5
R.m.s deviations			
Bond lengths (Å)	0.004	0.004	0.004
Bond angles (°)	0.601	0.632	0.607