

Persulfide dioxygenase from *Acidithiobacillus caldus*: Cysteine glutathionylation and identification of essential active site residues by mutation analysis

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Supplementary Information 2: File Ruhl_Supplement2_Alignments. Sequence alignments in FASTA format of the three subtypes of PDO based on the sequence set used by Xia *et al.* (2017)

Table S1: Oligonucleotides used in this study.

Primer name	Primer sequence (5'→3') ¹	Restriction enzyme	Purpose
<i>Acical_ETHE1_fwd</i>	aaaaatctagataacgaggcaaaaac- ATGTTATTCAAGCAGCTTTTTGACACCGA	<i>XbaI</i>	Amplification and ligation of the <i>pdo</i> gene with the pASK75 vector
<i>Acical_ETHE1_rev</i>	GCTCTCGAGTTCATGTGGATTGCTCCCGA- TGTCGTC		
<i>ask1b_fwd</i>	AGAGTTATTTTACCACTCCCTATCAG	-	Sequencing of vector constructs and mutants
<i>ask2_rev</i>	GCGTGGAGATCCGTGACGCA		
<i>AcPDO_C87A_fwd</i>	gcTGCaGACCGCAAGGTGG	<i>PstI</i>	
<i>AcPDO_C87A_rev</i>	ATCGGCTCCCGCGGC		
<i>AcPDO_C117A_fwd</i>	gcCGTGAGCTATCGtTGGCAC	<i>SmaI</i>	
<i>AcPDO_C117A_rev</i>	<u>CCcGGg</u> CGTGTGGCCGGGTGT		
<i>AcPDO_C117S_fwd</i>	aGtGTGAGCTATCGCTGGCAC	<i>BsaI</i>	Mutagenesis of cysteine codons in the <i>pdo</i> gene of <i>A. caldus</i>
<i>AcPDO_C117S_rev</i>	<u>cCCCGGCGTGTGGCCG</u>		
<i>AcPDO_C137A_fwd</i>	gcCGGcCGTACCGACTTTCAG	<i>EagI</i>	
<i>AcPDO_C137A_rev</i>	GCCGCCAATGAGCAGAGCGTC		
<i>AcPDO_C180A_fwd</i>	gcgATCGCCGAAGAGAAACGCAGT	<i>MboI</i>	
<i>AcPDO_C180A_rev</i>	ACTGACCCAGCGCCCGTGATA		
<i>AcPDO_C224A_fwd</i>	gcCGGtCGtGAtGACATCGGGAGC	Δ <i>NotI</i>	
<i>AcPDO_C224A_rev</i>	ACGCACATTAGCAGGGACCGCTACGTG		
<i>AcPDO_R139A_fwd</i>	gcTACCGACTTTCAGGGCGGC	<i>MwoI</i>	
<i>AcPDO_R139A_rev</i>	<u>gCCGCAGCCGCCAATGAG</u>		
<i>AcPDO_Y173A_fwd</i>	gcTCACGGcCGCTGGG	<i>EagI</i>	Mutagenesis of codons of putative substrate binding amino acid residues in the <i>pdo</i> gene of <i>A. caldus</i>
<i>AcPDO_Y173A_rev</i>	GTCGTGCCCGGGATAGACCAAG		
<i>AcPDO_P211A_fwd</i>	AAACACATaCACGTAGCGGTCCCC	<i>AflIII</i>	
<i>AcPDO_P211A_rev</i>	GGcTTGGGCCAGATCCAGG		
<i>AcPDO_K212A_fwd</i>	gcACACATaCACGTAGCGGTCCC	<i>AflIII</i>	
<i>AcPDO_K212A_rev</i>	GGGTTGGGCCAGATCCAGGG		
<i>AcPDO_T13A_fwd</i>	gCCTACACCTACATCCTGGGCG	<i>TaqI</i>	
<i>AcPDO_T13A_rev</i>	GCTGCTCTCGGTGTCgAAAAGCTG		
<i>AcPDO_T56A_fwd</i>	gCCCACGTCCATGCGGACCAC	<i>AflIII</i>	
<i>AcPDO_T56A_rev</i>	TTCCA <u>AcGCGT</u> AGCGCAGGGTC		
<i>AcPDO_H59A_fwd</i>	gcaGcTgACCACGTCAGCG	<i>PvuII</i>	
<i>AcPDO_H59A_rev</i>	GACGTGGGTTTCCAAAGCGTAGC		
<i>AcPDO_D61A_fwd</i>	GcaCACGTICAGCGCTGC	<i>AflIII</i>	Mutagenesis of codons of putative amino acids involved in the hydrogen bond network around the active site and the iron atom in the <i>pdo</i> gene of <i>A. caldus</i>
<i>AcPDO_D61A_rev</i>	CGCATGGACGTGGGTTTCC		
<i>AcPDO_H62A_fwd</i>	gcCGTCAGCGCTGCC	<i>RsaI</i>	
<i>AcPDO_H62A_rev</i>	GTCCGCATGtACGTGGGTTTCC		
<i>AcPDO_T110A_fwd</i>	gCaCCtGGCCACACGCCG	<i>MscI</i>	
<i>AcPDO_T110A_rev</i>	TGCCAGTACGCGGATGGC		
<i>AcPDO_T128A_fwd</i>	gCcGGCGACGCTCTGCTCATTG	<i>MspI</i>	
<i>AcPDO_T128A_rev</i>	GAATACGCGATCGTGCCAGCGATAG		
<i>AcPDO_H171A_fwd</i>	TATCACGGGCGCTGGGTCAG	<i>NarI</i>	
<i>AcPDO_H171A_rev</i>	GTCGgcgCCGGGATAGACC		
<i>AcPDO_H57A_fwd</i>	gcCGTgCATGCGGACCACaTCAGCGCTG	<i>SphI</i>	
<i>AcPDO_H57A_rev</i>	cGTTTCCAAAGCGTAGCGCAGcGTCAG		
<i>AcPDO_H57G_fwd</i>	ggCGTCCATGCGGACCAC	<i>MwoI</i>	
<i>AcPDO_H57G_rev</i>	GGTcTCCAAAGCGTAGCGCAGG		
<i>AcPDO_H113A_fwd</i>	gcCACGCCGGGCTGCGTGAGCTATC	<i>NarI</i>	
<i>AcPDO_H113A_rev</i>	GCCGGGTGTTGCCAGTACGCGGATG		

¹Restriction sites underlined; non-complementary nucleotides in lower case

Table S1 continued: Oligonucleotides used in this study.

<i>Ac</i> PDO_H113G_fwd	<u>ggCACGCCGGGCTGC</u>	<i>MwoI</i>	
<i>Ac</i> PDO_H113G_rev	aCCGGGTGTTGCCAGTACGC		
<i>Ac</i> PDO_D130A_fwd	<u>GcCGCTCTGCTCATTGGCGGCTGCGG</u>	<i>NarI</i>	Mutagenesis of codons of primary iron ligands in the <i>pdo</i> gene of <i>A. caldus</i>
<i>Ac</i> PDO_D130A_rev	GCCgGTGAATACGCGATCGTGCCAG		
<i>Ac</i> PDO_D130E_fwd	<u>GAaGCTfTGCTCATTGGCGGCTGCGG</u>	<i>HindIII</i>	
<i>Ac</i> PDO_D130E_rev	GCCgGTGAATACGCGATCGTGCCAG		
<i>Ac</i> PDO_D130H_fwd	<u>cACGCTCTGCTCATTGGC</u>	<i>MscI</i>	
<i>Ac</i> PDO_D130H_rev	<u>GCCaGTGAATACGCGAGC</u>		

¹Restriction sites underlined; non-complementary nucleotides in lower case

Table S2: Overview of specific activities of wild type *Acidithiobacillus caldus* PDO (*Ac*PDO) and its variants, iron content, numbers of preparations including total number of assays and T_m values.

	Specific activity	Fe loading	Protein	# Preps # Assays	T _m	# Preps # Assays
	<i>U/mg</i>	<i>mol/subunit</i>	<i>mg/L medium</i>		<i>°C</i>	
Wt	61.6 ± 3.5	0.77 ± 0.10	26.3	18 39	63.0 ± 1.3	5 15
T _{13A}	31.5 ± 3.9	0.80 ± 0.04	38.9	1 3	n.d. ²	-
T _{56A}	-	-	- ¹	3 0	-	-
H _{59A}	4.1 ± 0.1	0.45 ± 0.02	25.1	1 3	n.d.	-
D _{61A}	≤ 0.05	0.53 ± 0.01	36.4	1 6	61.0 ± 0.05	1 3
H _{62A}	≤ 0.05	0.76 ± 0.05	38.2	1 6	63.3 ± 0.05	1 3
C _{87A}	1.2 ± 0.1	0.73 ± 0.05	39.2	2 6	63.3 ± 1.4	3 8
T _{110A}	34.9 ± 2.1	0.75 ± 0.03	25.5	1 3	n.d.	-
T _{128A}	18.5 ± 1.9	0.39 ± 0.05	10.8	1 3	n.d.	-
C _{117A}	6.3 ± 0.3	0.65 ± 0.06	15.6	3 9	n.d.	-
C _{117S}	41.8 ± 3.2	0.86 ± 0.07	34.2	1 3	n.d.	-
C _{137A}	33.5 ± 2.0	0.81 ± 0.05	31.2	2 6	n.d.	-
R _{139A}	0.3 ± 0.04	0.78 ± 0.02	33.5	4 9	60.7 ± 0.8	1 3
D _{141A}	1.5 ± 0.2	0.50 ± 0.01	34.9	1 3	55.7 ± 0.2	1 3
H _{171A}	≤ 0.05	0.74 ± 0.20	26.2	3 9	54.9 ± 0.7	1 3
Y _{173A}	6.6 ± 0.7	0.68 ± 0.02	26.2	3 6	n.d.	-
C _{180A}	54.6 ± 4.7	0.79 ± 0.01	28.5	1 3	n.d.	-
P _{211A}	23.6 ± 0.6	0.84 ± 0.04	34.4	3 6	n.d.	-
K _{212A}	5.8 ± 0.4	0.88 ± 0.04	38.5	3 6	n.d.	-
C _{224A}	0.7 ± 0.06	0.97 ± 0.05	32.1	2 6	63.3 ± 0.05	2 6

¹No protein was obtained

²n.d., not determined

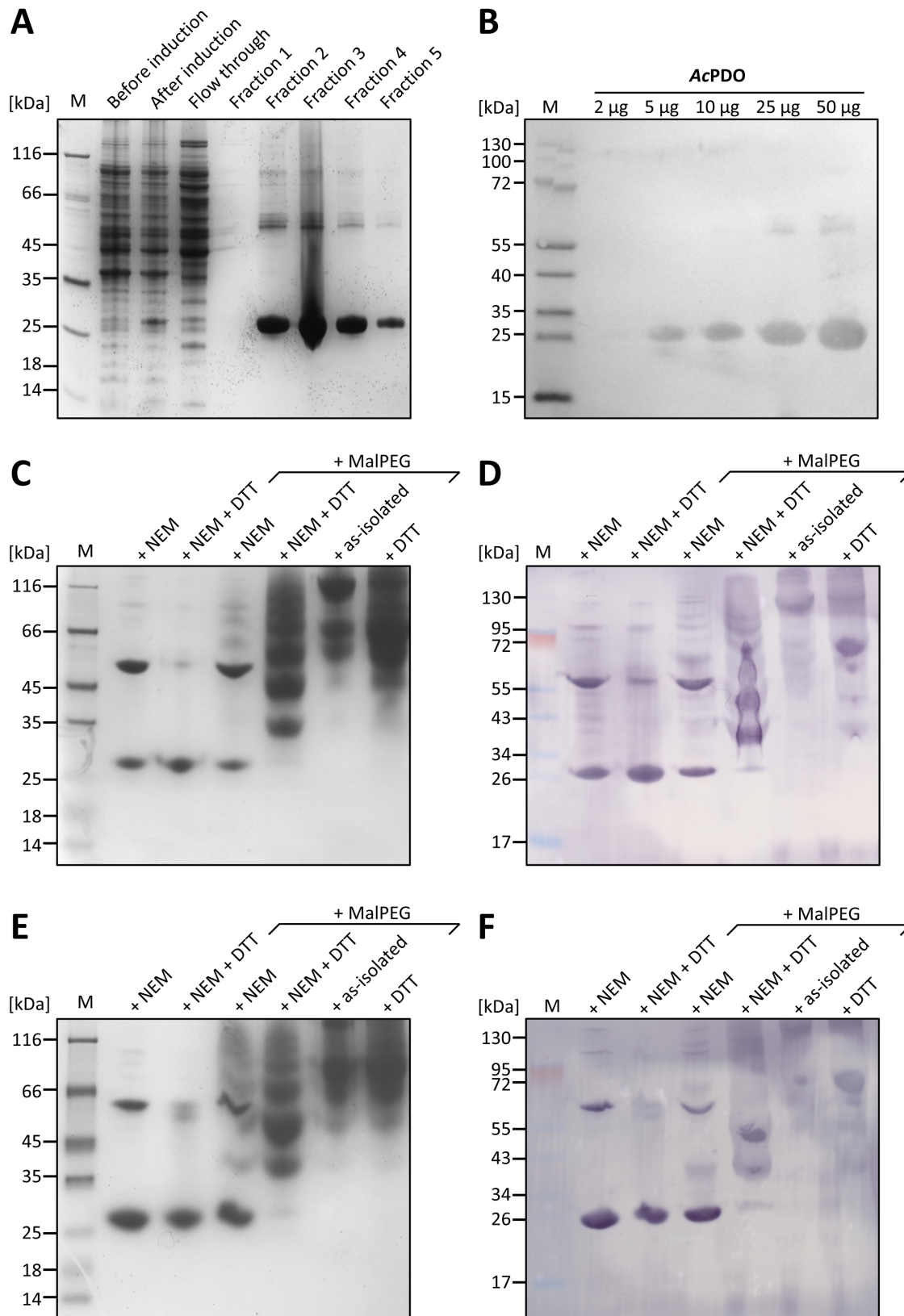


Figure S1. SDS Gel, MalPEG gel shift assays and Western analyses of the wild type and C₈₇ and C₂₂₄ variants of the *Acidithiobacillus caldus* PDO. **A**, 10% Tris-tricine polyacrylamide gel (Schägger and von Jagow, 1987) with total cell extracts before and after induction (from 100 μ l of the culture); the flow-through of the Streptag column of the ultracentrifuge supernatant after cell disruption and the elution fractions 1-5 (10 μ l each). **B**, Western analysis of increasing amounts of AcPDO hybridized with horseradish peroxidase-coupled Streptactin; M, marker proteins.; **C**, Coomassie-stained MalPEG gel shift assay of the AcPDO C₈₇A variant and **D**, same with Western analysis using StrepMAP-Classic HRP-conjugated antibody (10-20 μ g/lane). **E**, **F**, same as in panels C and D with the AcPDO C₂₂₄A variant.

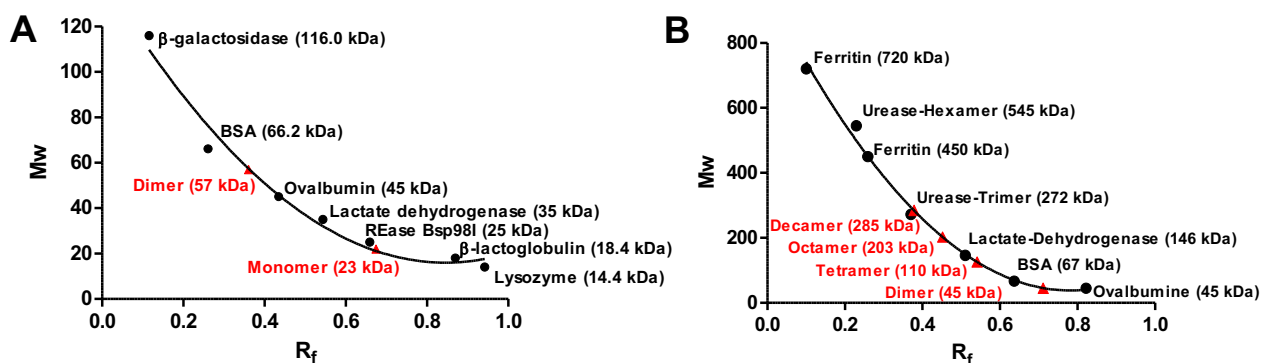


Figure S2. Calibration plot of R_f values of denaturing (A) and non-denaturing (B) polyacrylamide gel electrophoresis.

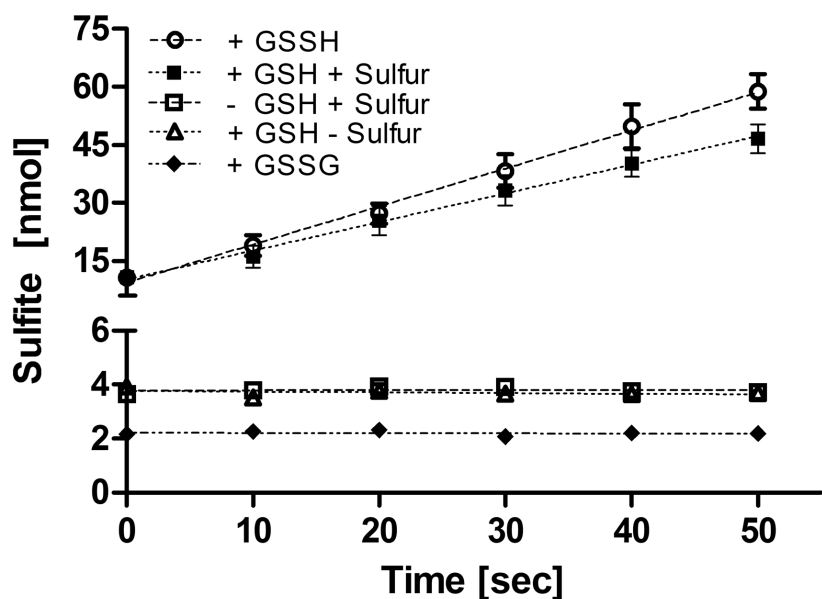


Figure S3. Activity profile of the *AcPDO*. Sulfite formation of the wild type *AcPDO* with 1 mM GSH, GSSH, or GSSG and/or 2 % elemental sulfur; 1 μg/ml of protein per assay and non-enzymatic controls.

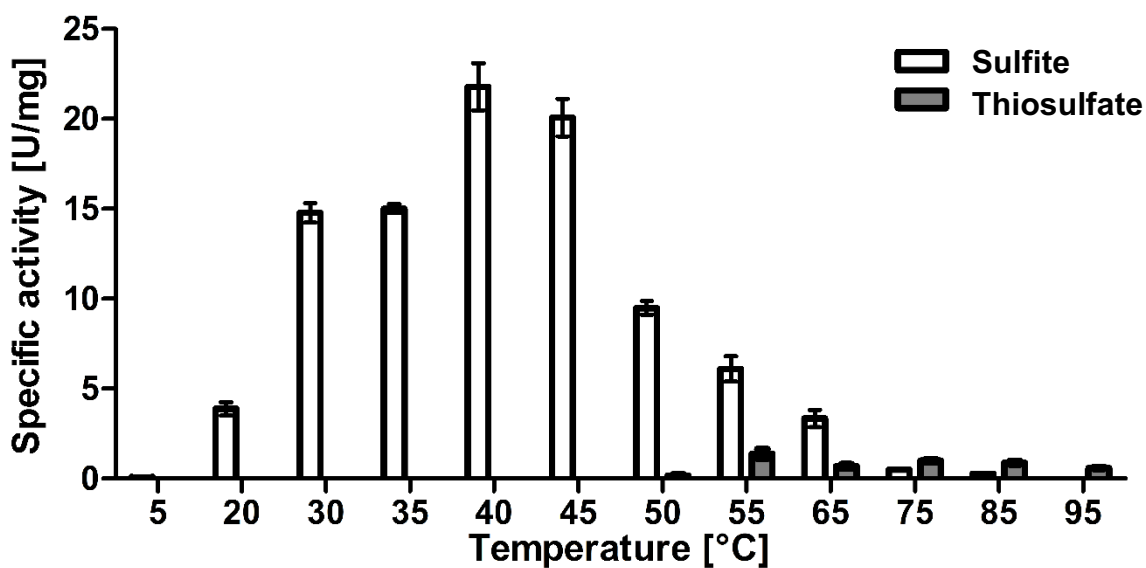


Figure S4. Formation of sulfite and thiosulfate during the enzyme reaction of the *Ac*PDO at pH 7.5 and different temperatures.

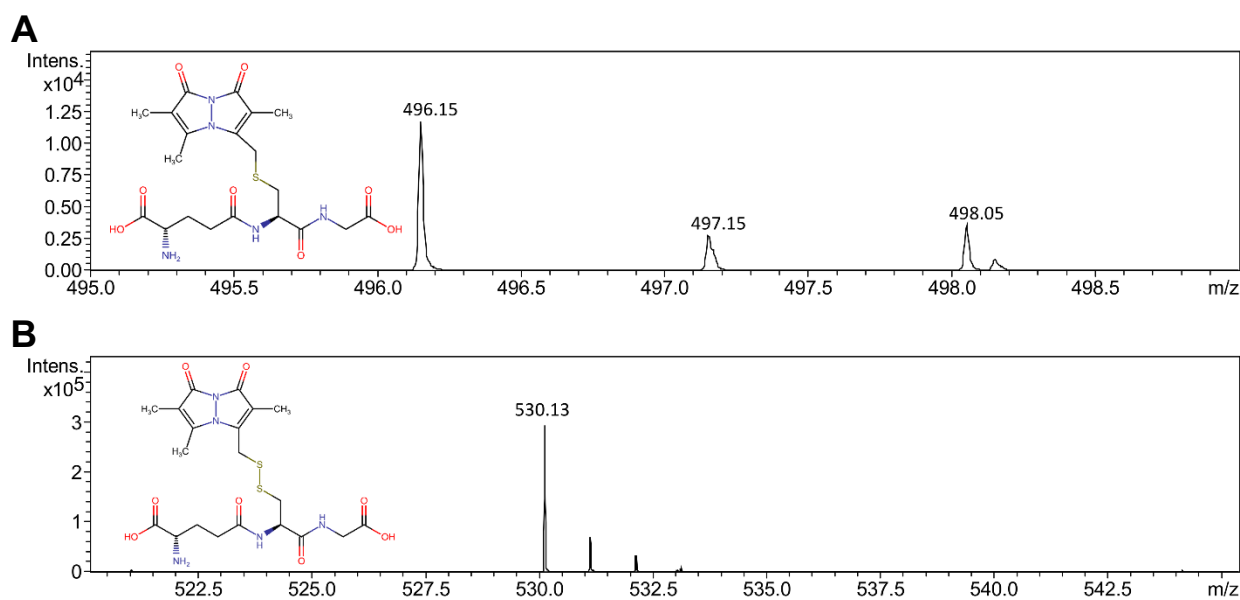


Figure S5. Mass spectra of GSH (A) and chemically synthesized GSSH (B) both derivatized with monobromobimane before analysis.

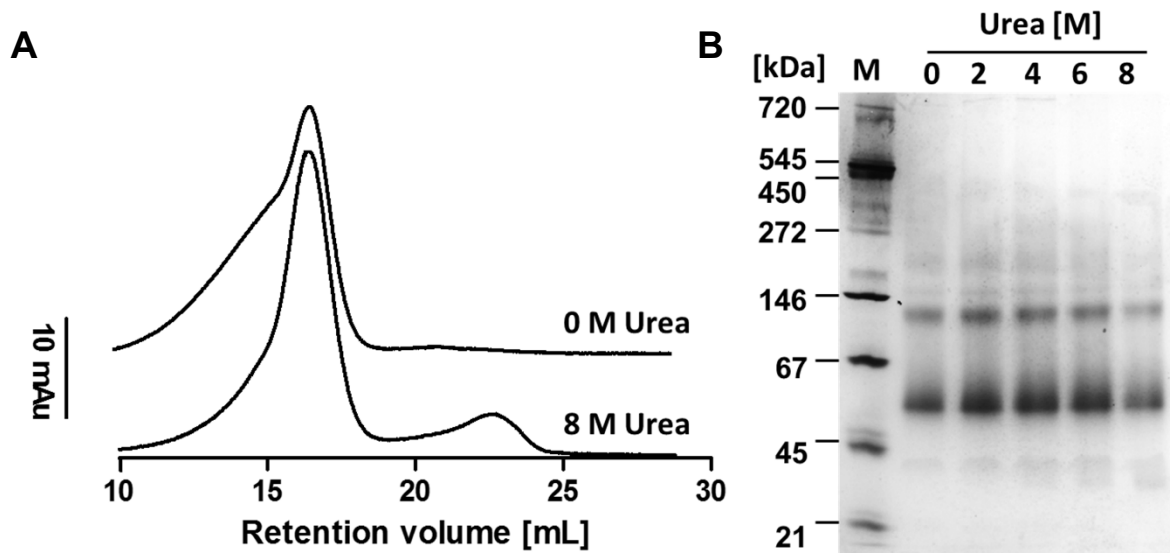


Figure S6. Urea denaturation experiments of wild type *AcPDO*. **A**, Merged gel permeation chromatograms of *AcPDO* before and after denaturation with 8 M urea. **B**, Native polyacrylamide gel after treatment of the *AcPDO* with different concentrations of urea.

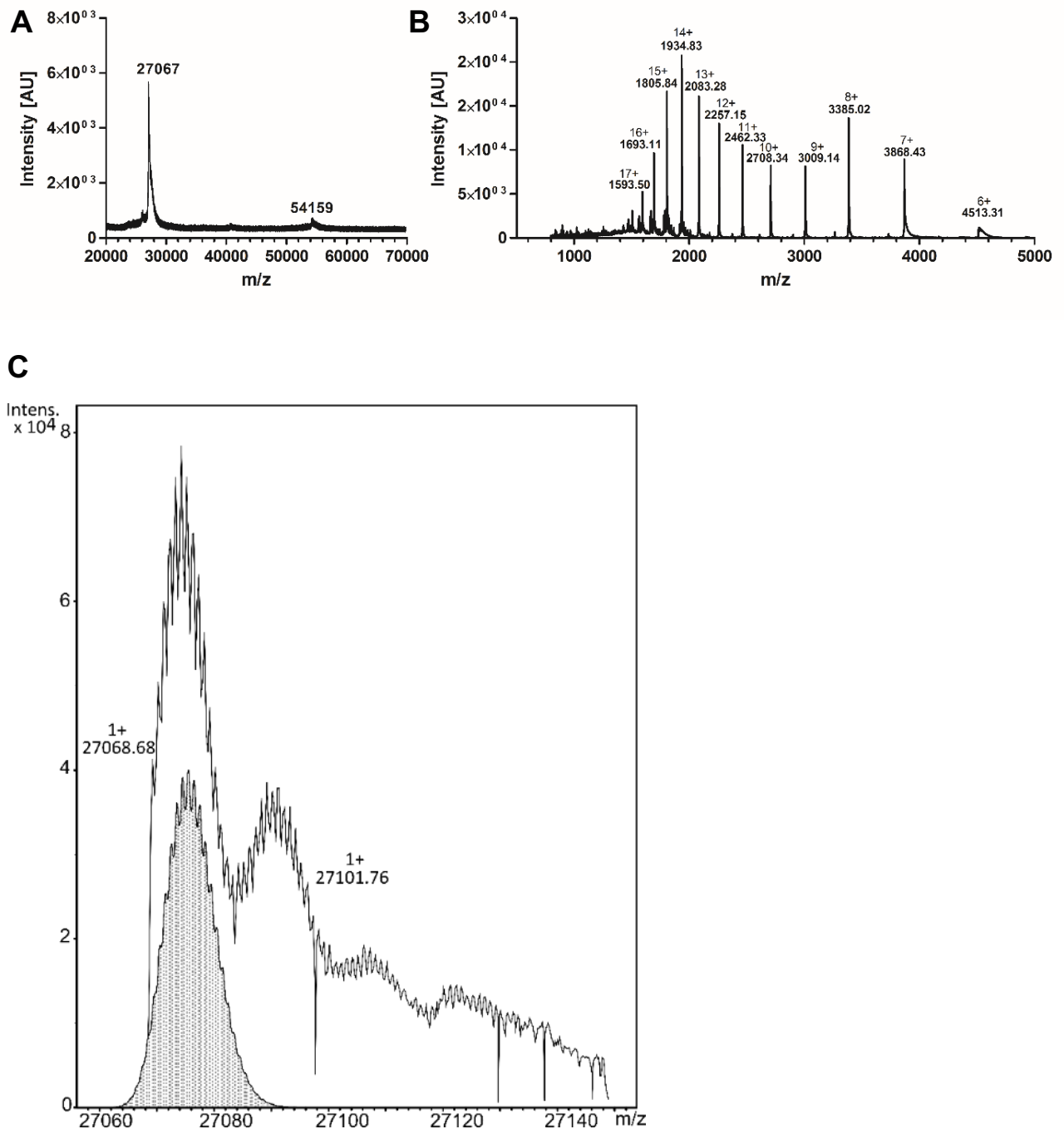


Figure S7. Mass spectra of the AcPDO holoenzyme. **A**, MALDI-TOF spectrum. **B**, ESI spectrum (positive ionization mode). **C**, line, deconvoluted ESI spectrum; grey shaded area, calculated spectrum.

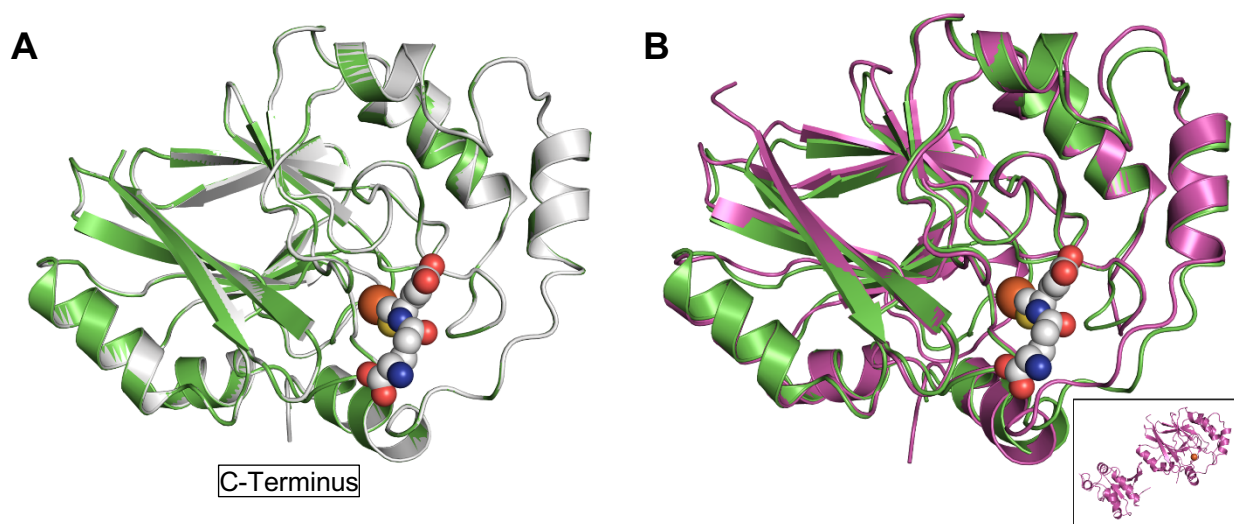


Figure S8. Comparison of the *Ac*PDO model with 3D structures of PDOs from *Myxococcus xanthus* (*Mx*PDO) and *Paraburkholderia phytofirmans* (*Pp*PDO). **A**, Comparison of the *Ac*PDO (green) with the *Mx*PDO (grey; PDB accession number: 4YSB) (Sattler et al., 2015). **B**, Comparison of the *Ac*PDO with the *Pp*PDO (purple; PDB accession number: 5VE5) (Motl et al., 2017); spheres, GSH and iron from *Pp*PDO structure; box, holoenzyme of the *Pp*PDO with C-terminally fused rhodanese domain.

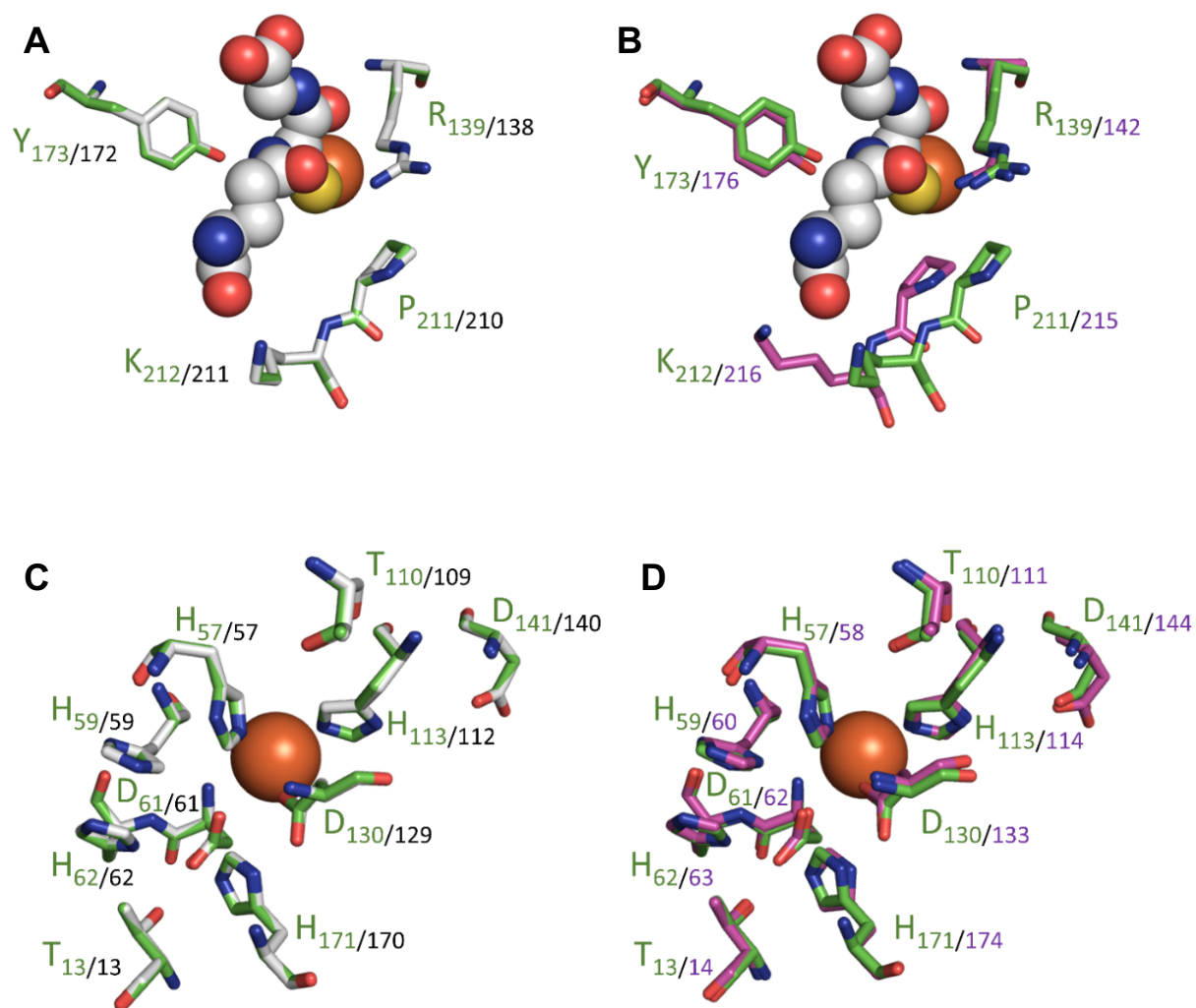


Figure S9. Structural comparison of the predicted active sites of the *Ac*PDO 3D model (green), *Mx*PDO (grey) and *Pp*PDO (purple) focussing on exchanges of amino acids putatively taking part in substrate binding (A, B) and in the hydrogen bonding network around the active site pocket (C, D).

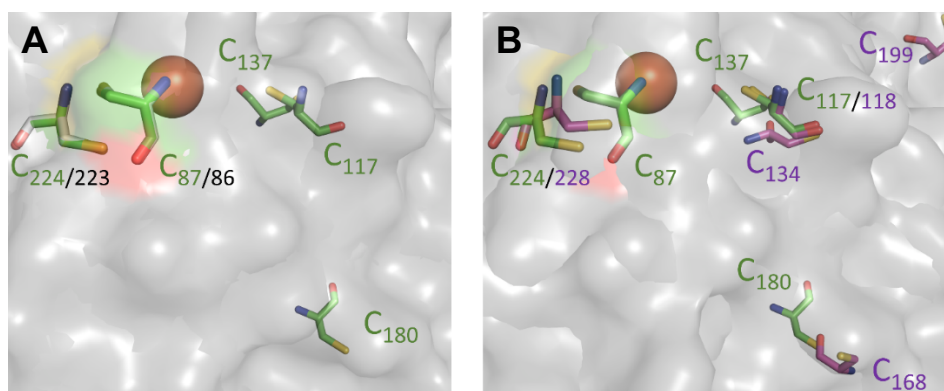


Figure S10. Structural comparison of cysteine residues between (A) the *Ac*PDO model (green) and *Mx*PDO (grey) and (B) the *Ac*PDO model (green) and the *Pp*PDO (purple).

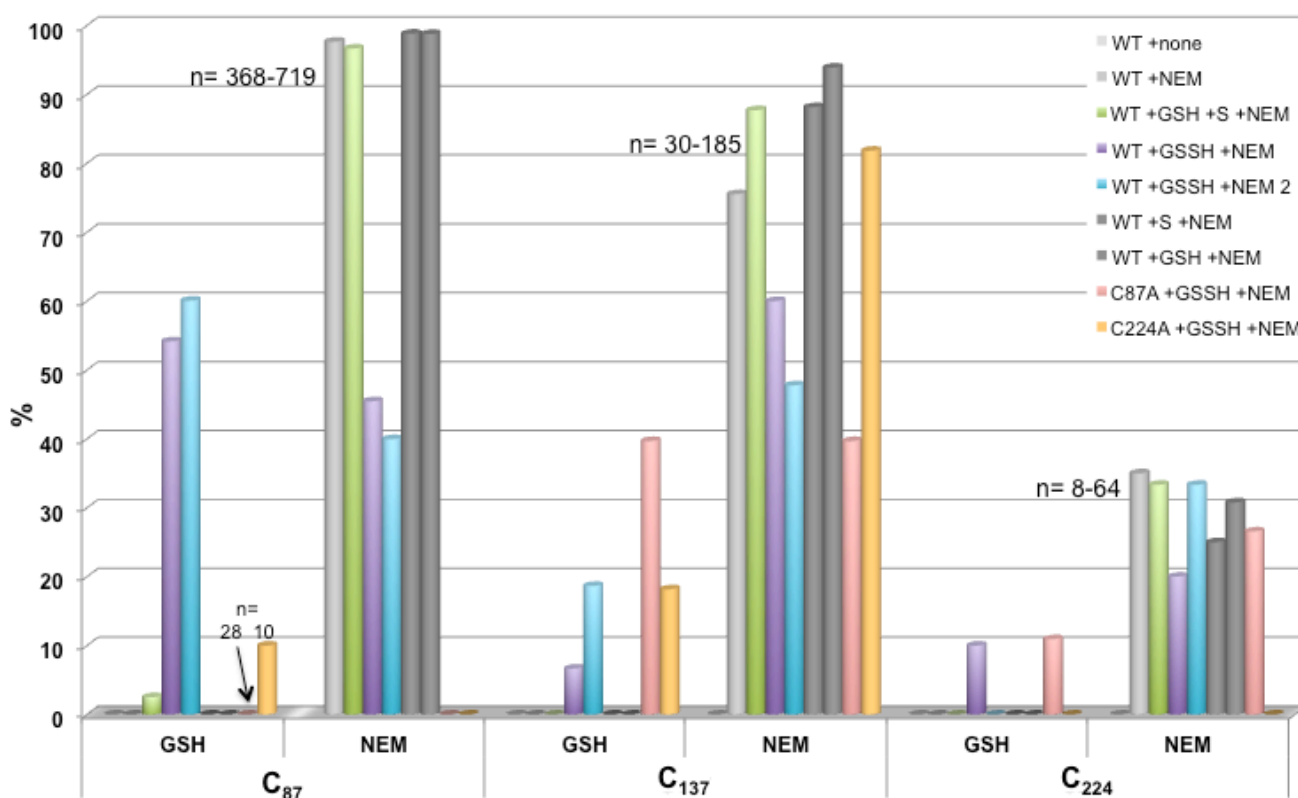


Figure S11. Relative abundances of cysteine modifications in MALDI-TOF mass spectrometry fingerprinting after treatment of the *Ac*PDO with various substrates for 1 min each in enzyme reaction buffer (70 mM Tris/HCl pH 7.5, 0.1% Tween20), followed by NEM derivatization; GSH and GSSH, each 1 mM; S, 2% sulfur flower; n represents the total number of fragment spectra recorded with cysteine modification; WT, wild type; C87A and C224A, cysteine variants.

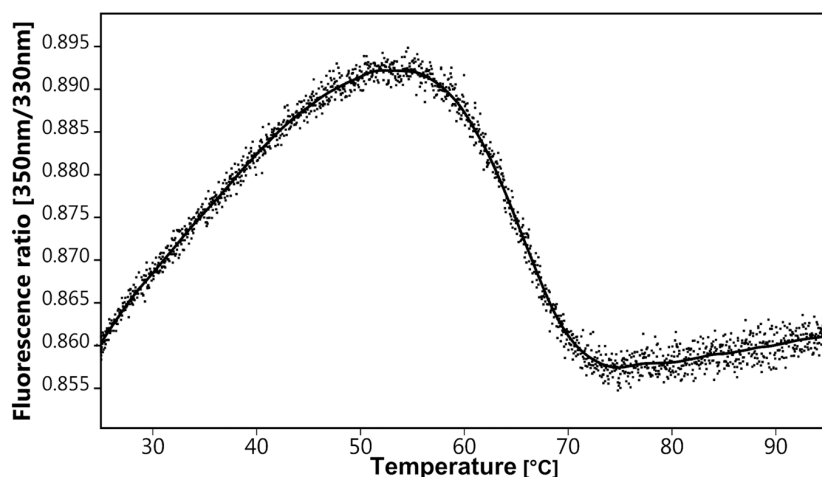


Figure S12. Differential scanning fluorimetry (tryptophane/tyrosine fluorescence) of wild type *AcPDO* (1 mg/ml), measured in buffer E at a heating rate of 1 K / min.

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