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Thiol Dioxygenase from Pseudomonas aeruginosa

The Cysteine Dioxygenase Homologue from *Pseudomonas aeruginosa* is a 3-Mercaptopropionate Dioxygenase*

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Background: Thiol dioxygenation is catalyzed by enzymes specific for each substrate.

Results: Kinetic, structural and spectroscopic data describes an enzyme from *P. aeruginosa* that is a 3-mercaptopropionate dioxygenase with secondary cysteine dioxygenase activity.

Conclusion: An arginine to glutamine switch and absence of a cis-peptide bond correlates with substrate preference.

Significance: Characterization of this enzyme deepens our understanding of substrate specificity in thiol dioxygenases.

ABSTRACT

Thiol dioxygenation is the initial oxidation step that commits a thiol to important catabolic or biosynthetic pathways. The reaction is catalyzed by a family of specific non-heme mononuclear-iron proteins each of which is reported to react efficiently with only one substrate. This family of enzymes includes cysteine dioxygenase, cysteamine dioxygenase, mercaptosuccinate dioxygenase and 3mercaptopropionate dioxygenase. Using sequence alignment to infer cysteine dioxygenase activity, a cysteine dioxygenase homologue from Pseudomonas aeruginosa (p3MDO) has been identified. Mass spectrometry of P. aeruginosa under standard growth conditions showed p3MDO is expressed in low levels suggesting that this metabolic pathway is available to the organism. Purified recombinant p3MDO is able to oxidize both

cysteine and 3-mercaptopropionic acid (3-MPA) in vitro, with a marked preference for 3-MPA. We therefore describe this enzyme as a 3mercaptopropionate dioxygenase. Mössbauer spectroscopy suggests that substrate binding to the ferrous iron is through the thiol but indicates each substrate could adopt different coordination geometries. Crystallographic comparison with mammalian cysteine dioxygenase shows that the overall active site geometry is conserved but suggests that the different substrate specificity can be related to replacement of an arginine by a glutamine in the active site.

The global sulfur cycle involves interconversion of sulfur through a wide range of oxidation states. The most abundant oxidation states of sulfur are -2, as thiol/ate (eg. cysteine or hydrogen sulfide), 0 as elemental sulfur, and +6 as sulfate. Within the sulfur cycle, microbiological transformation dominates the conversion of inorganic sulfur and to some extent organic sulfur.(1) While bacteria are very flexible in terms of their sulfur source, animals require dietary intake of sulfur-containing amino-acids such as methionine, which serves as a biosynthetic precursor of cysteine.(2) In organisms, sulfur is present in proteins, nucleic acids, sulfate esters of polysaccharides, steroids, phenols, and sulfurcontaining coenzymes.(3,4) Therefore, sulfurdependent enzymology represents an important means of biological sulfur utilization. In particular, two thiol-containing compounds, cysteine and 3mercaptopropionic acid, are of interest as they are both abundant and are irreversibly removed during their respective metabolic cycle by dioxygenation.(2,5-7)

In mammals, cysteine levels are maintained directly from dietary sources and/or through biosynthesis from methionine.(2,7) High cysteine levels have been implicated in a number of neurodegenerative diseases due to its exitotoxic effect in the nervous system.(8-12) Accumulation of cysteine has been attributed to impaired function of cysteine dioxygenase (CDO)[†],(9,13-15) an enzyme catalyzing dioxygenation of cysteine thiolate to sulfinate (CSA).(16-21) Functional CDO occurs widely and has been identified in mammals, lower eukaryotes and in bacteria.(21-23) CDO belongs to a family of nonheme mononuclear-iron dioxygenases, but stands out due to a number of unique characteristics. The iron atom has His₃ coordination and, in forms. post-translational mammalian а modification involving a cysteine-to-tyrosine crosslink is present.(24,25)

3-mercaptopropionic acid (3-MPA) is a structural analogue of cysteine (Fig 1), lacking only the amine group. In spite of the fact that it is one of the most abundant thiols in wetlands(26)and fresh water lakes(27) most bacteria cannot use 3-MPA as a sole carbon source.(28-30) Instead, bacteria are capable of growing on various 3-MPA precursors, which are catabolized to 3-MPA inside the cell.(28-31) In bacteria, 3-MPA is a central metabolite in both catabolic and assimilatory sulfur metabolism (5) and while bacteria can form 3-MPA as a metabolite of methionine. homocysteine (32)or dimethylsulfoniopropionate,(31-33) little is known about the dissimilation of 3-MPA. In one Variovarax paradoxus, bacterium, 3-MPA utilization was shown to involve dioxygenation of 3-MPA to 3-sulfinopropionic acid (3-SPA), catalvzed CDO homologue, bv а 3mercaptopropionate dioxygenase (3MDO) which showed high specificity for 3-MPA but not for cysteine.(6) Interestingly, 3-MPA acts as an inhibitor of metallo-β-lactamase, an enzyme involved in antibiotic resistance in bacteria.(34,35) In mammals 3-MPA is toxic by inhibiting the oxidation of fatty acid(36,37) and the synthesis of the inhibitory neurotransmitter γ -aminobutyric

acid, resulting in deformation, seizures or death of rats.(38-40)

The gram-negative proteobacterium Pseudomonas aeruginosa is an opportunistic pathogen, which can infect plants and animals, including humans. This bacterium has gained importance as a nosocomial pathogen that infects immune-compromised patients and is highly resistant to antibiotic treatment.(41) Due to its ability to utilize a wide range of organic matter and cope with different environmental conditions this organism can colonize many natural environments including soil, water and skin. By implication, P. aeruginosa metabolic enzymology illustrates various means of environmental adaptation. Considering that P. aeruginosa colonizes human hosts it is not surprising that it can utilize cysteine or methionine as a sulfur source.(42) Although it has been established that cysteine can be fully reduced to H₂S in the course of P. aeruginosa metabolism.(43) oxidation of cysteine to CSA through CDO or an equivalent enzyme has not been reported. Similarly, little is known about bacterial enzymes involved in 3-MPA metabolism. Strains from the Pseudomonas family are capable of 3-MPA utilization.(28) Conversion 3-SPA 3SP-CoA of to in Proteobacteria has been attributed to succinylsynthetase.(44.45) however enzymatic CoA oxidation of 3-MPA to 3-SPA remains to be identified. CDO homologues from bacteria (Ralstonia eutropha, PDB accession 4QMA (2GM6), Bacillus subtilis, 3EQE) are structurally very similar to mammalian CDO, for which several X-ray crystal structures are available, but the substitution of glutamine in bacterial enzymes for arginine at position 60 of the mammalian active site suggests altered substrate specificity.

In this study we report identification, classification, structural, kinetic and spectroscopic *P*. characterization of aeruginosa thiol dioxygenase (p3MDO). This enzyme is a 3-MPA dioxygenase with secondary cysteine dioxygenase activity. This is the first report of enzymology of cysteine and 3-MPA dissimilation in P. aeruginosa metabolism and the first example of a CDO homologue which utilizes a second substrate with near stoichiometric coupling to dioxygen consumption.

Experimental Procedures

Thiol Dioxygenase from Pseudomonas aeruginosa

Protein Sequence Identification, Expression and Purification. The NCBI reference sequence (NC 002516.2) for *P*. aeruginosa (strain PAO1)(46) contains region а gi|110645304:2945264-2945869 coding for a hypothetical protein PA2602 (NP 251292.1).(47) BLAST search(48) of putative bacterial CDO sequence (YP 299237.1) from R. eutropha (strain JMP134) was performed using the SIB-BLAST network service and identified PA2602 as a CDO homologue (Fig 2). The experimental design for the PCR-amplification of the PA2602 coding sequence was optimized using Primer D'Signer software (IBA). DNA sequence coding for CDO was PCR-amplified using Pfu DNA polymerase (Stratagene) and inserted into pPR-IBA1, a Streptag affinity-based expression plasmid (IBA) digested with BsaI (Fermentas), according to standard procedures to yield an open reading frame encoding the full PA2602 protein with a Cterminal extension of SAWSHPOFEK. Both strands of the coding region of the P. aeruginosa PA2602 expression construct (pPR-IBA1/PA2602/P. aeruginosa) were fully sequenced at the Genetic Analysis Services at the University of Otago to confirm the absence of adventitious mutations. A molecular weight of 23746.5 Da was calculated based on the amino acid sequence of the expressed protein.(49,50) PA2602 expression was induced in BL21(DE3)pLvsS cells (Novagen) and purified using Strep-tag affinity chromatography according to the manufacturer's protocol (IBA).(51,52) We employed the following modifications to the standard protein expression/purification procedures: (i) cells were incubated overnight at 18 °C during protein expression, (ii) cell lysis buffer contained 100 mM TRIS (pH=8.0), 1.15 M NaCl, 1mM EDTA, 5 mM Tween 20 (1.5% v/v) and a Roche complete protease inhibitor, and (iii) cells were lysed using a French pressure cell (model FA-078, SLM Aminco). The purified protein preparations were dialyzed extensively $(>10^9$ dilution factor) against buffer compatible with the subsequent experiment. Protein was concentrated on Vivaspin centrifugation concentrators (GE Healthscience) to the appropriate concentrations for the subsequent experiments. As expected, P. aeruginosa CDO homologue preparations resolved into a single band when subjected to SDS-PAGE revealing the

absence of a posttranslational modification present in mammalian CDO(53-56) (Fig 3). Protein preparations were found to be at least 97.5% pure and contained ~15% endogenously bound iron as judged by colorimetric assay using ferrozine in a 96-well format as previously described.(57) Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 29700 M⁻¹cm⁻¹ estimated from a bovine serum albumin standard curve quantified by SDS-PAGE densitometry. For anaerobic studies protein preparations, corresponding buffers and solvents were made anaerobic by alternating cycles of vacuum and purging with argon gas on a Schlenk line kept on ice slurry. All anaerobic manipulations were performed in a nitrogen atmosphere in a glovebox (Belle Technology, <1 ppm oxygen).

Assessment of PA2602 protein expression in Pseudomonas aeruginosa. P. aeruginosa strain PAO1 was grown in L-broth to late logarithmic phase (OD600 \sim 3.3) and the cells collected by centrifugation. Approximately 0.6 g cell pellet was re-suspended in 2.3 mL 100 mM phosphate buffer pH 7.5 in the presence of protease inhibitors. Cells were sonicated (SONICS Vibra cell) on ice for 5 min (9 s on, 9 s off, amplitude 38%). The lysed cells were spun down at 30,000 g at 4 °C for 30 min. 100 uL of the supernatant were mixed with 500 µL ice-cold acetone and stored at -20 °C for 45 min to precipitate the proteins. After spinning the solution for 30 min at 16,000 g at room temperature the supernatant was discarded and the pellet air-dried on the bench overnight. The dried pellet was dissolved in SDS-buffer and proteins were separated by SDS-gel electrophoresis. Comparison to the retention of purified p3MDO on a separate gel and with the use of molecular weight markers a region of interest of about 1 cm between 21.5 and 31 kDa was excised from the gel to be measured by mass spectrometry. The proteins were digested 'in gel' with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi, Intavis AG, Cologne, Germany) essentially following the protocol as described by Shevchenko et al.(58)

Parallel reaction monitoring (PRM) assays were performed by nanoflow uHPLC-coupled TripleTOF 5600+ mass spectrometry (AB Sciex, Framingham, MA). The m/z values of doubly and triply charged precursor ions and their 10 strongest

singly charged transitions (m/z of fragment ions) were predicted for seven proteotypic peptides of p3MDO using the skyline software (https://skyline.gs.washington.edu).(59) An aliquot of the sample was first analyzed by an unscheduled PRM assay using the target peptides given in Table 1.

LC separation of peptides was performed on an Ekspert 400 LC-system (Eksigent, AB Sciex) at a flow rate of 500 nL/min using a homemade emitter tip column (75 µm ID PicoTip fused silca tubing (New Objectives, Woburn, MA) packed with 3 µm C-18 beads on a length of 12 cm). The gradient was developed from 2% acetonitrile, 0.2% formic acid in water to 25% acetonitrile, 0.2% formic acid in water over 45 min followed by an increase to 90% acetonitrile, 0.2% formic acid in water over 15 min. The mass spectrometer was operated in precursor mode with a precursor isolation window of 0.7 units (unit-resolution) and an ion accumulation time of 100 ms to allow for the acquisition of both charge states of all 7 precursors (14 ions per cycle) within a 2 second cycle time. For precursor fragmentation rolling collision energy was selected and all transitions were monitored in parallel by the TOF analyzer in a high resolution fragment-ion spectrum. Raw data of the unscheduled PRM were imported into the skyline software for compound detection and determination of retention times. Skyline was then used to build a scheduled PRM method of only the strongest detected charge state of each precursor within a retention time window of 2 min to be used by the instrument software (Analyst software, AB Sciex). For the scheduled PRM method the same LC-gradient and MS-settings were used as for the unscheduled PRM with the exception of an increased ion accumulation time of 150 ms for each precursor and a maximum of 4 precursors per cycle resulting in a cycle time of 0.9 sec.

Raw data were analyzed using Skyline for compound detection and the peak view software (AB Sciex) for the extraction of high resolution fragment-ion spectra and peak lists. Peak lists were converted to a Mascot generic format for software-assisted sequence assignment by the database-dependent search engine Mascot (matrixscience.com). Spectra were searched against the non redundant NCBI amino acid sequence database (48,573,147 sequence entries) allowing for the identification of semi-tryptic peptides with a maximum of 3 missed cleavage sites and the variable modifications of carboxyamidomethylated cysteine, oxidized methionine and deamidated asparagine/glutamine. Precursor and fragment ion accuracy were 25 ppm and 0.1 Da respectively.

Crystallization and Structure Determination. Crystals were grown using protein that had been reconstituted with iron (see below) by both the hanging-drop and the sitting-drop vapor diffusion methods. To prepare hanging-drops, 300 nL of protein at 11 mg/mL in water was mixed with 150 nL of 100 mM HEPES, 100 mM NaCl, 1.6 M (NH₄)₂SO₄, pH=7.5. Crystals grew as extended rhombohedra of ~100 µm at 16 °C in less than 14 days. Crystals were transferred to a 15% (v/v) solution of glycerol in the well solution and mounted in a loop at -180 °C. Sitting-drop crystals were produced in 70 μ L reservoirs by mixing 2 μ L protein (11 mg/mL) with 1 µL mother liquor containing 200 mM sodium acetate, 8% (w/v) PEG 4000, pH=5.7. Crystals grew quickly as extended rhombohedra of ~500 µm within days at 18 °C and these were transferred to 25% (v/v) ethylene glycol in well solution and mounted in a loop at -180 °C.

Using a crystal A, grown from ammonium sulfate, 180° of data were collected using 1° oscillations at the MX1 and MX2 beamlines of the Australian synchrotron with a wavelength of 0.9537 Å and an Area Detector Systems Corporation (ADSC) detector. Data were indexed in space group $P2_12_12_1$, with unit cell dimensions of 184.38, 63.02 and 35.49 Å, integrated in Mosflm and scaled using Scala; 5% of reflections were reserved for the calculation of the R_{free} statistics (Table 2). Programs of the CCP4 suite (The CCP4 suite, 1994) were used for structure solution. Phases were solved by molecular replacement using the R. eutropha structure (2GM6, modified in Chainsaw) as a search model in Phaser.(60) Two monomers were placed in the asymmetric unit. Automated model refinement was performed in Refmac 5,(61) with two TLS groups defined in TLSMD (62) and medium NCS restraints. Models were inspected in COOT(63) and manual adjustment performed to match chemical expectations, electron density maps and anomalous electron density maps (this last for iron atoms only). Electron density adjacent to the active site iron was modeled as solvent molecules,

including a sulfate ion, in agreement with other CDO structures from crystals prepared in the presence of sulfate and absence of substrate and product. However, the resolution was not sufficient to rule out other explanations such as bound product (CSA) carried through the purification. A final round of refinement in Refmac5.5 used diffraction data to 2.7 Å but no prior phase information (Table 2).

A preliminary data set of 180° at 1° oscillation were collected at MX2 of the Australian Synchrotron from sodium acetate grown crystal B and indexed in space group $P4_12_12$, with a unit cell dimensions of 66.94, 66.94, 377.35 Å. Data were integrated to 2.5 Å in XDS and scaled using Scala; 5% of reflections were reserved for the calculation of the R_{free} statistics (Table 2). The structure obtained using crystal A was used for molecular replacement yielding four monomers in the asymmetric units. A higher resolution data set was collected by the same method from a second sodium acetate grown crystal (crystal C), and indexed with parameters indistinguishable from those for the preliminary data set from crystal B. The programs of the CCP4 suite and Phenix were used for structure solution, with the refined 2.5 Å structure from crystal B used for molecular replacement. Four monomers were placed in the asymmetric unit. The active site iron and solvent molecules were placed using Phaser-EP (MR-SAD). Phenix Refine and Readyset were used for refinement. The following residues were not included in the final model (chain A: 1-4, 199-211; chain B: 1-4, 198-211; chain C: 1-3, 200-211; chain D:1-3, 202-211).

Iron Binding by PA2602 Protein. Iron binding was investigated by adding various amounts of ferrous iron sulfate to deoxygenated protein, removing the excess iron by Chelex 100, and then measuring the protein-bound iron by ferrozinebased assay.(57) The data were fitted to(64,65): y = $0.5(K_{app}+E+x) - (0.25(K_{app}+E+x)^2 - Ex)^{1/2}$, where x is the concentration of added iron, y is the concentration of detected protein-bound iron, E is the concentration of protein competent for iron binding and K_{app} is the apparent dissociation constant for the protein-iron complex.

Cysteine-dependent Oxygen Depletion Activity of *PA2602 Protein.* Activities upon addition of cysteine to protein samples were tested by following oxygen depletion with a Clarke-type oxygen electrode (Rank Brothers) as previously described.(57) Appropriate control experiments were conducted to confirm that oxygen depletion was not due to cysteine auto-oxidation under reaction conditions.

Quantitative Assessment of Cysteine Dioxygenase activity of PA2602 Protein after Fe^{II} Enrichment. PA2602 cysteine dioxygenase activity after treatment of the protein with ferrous iron sulfate followed by Chelex was measured using HPLC-Evaporative light scattering detector (ELSD) with a Luna 5 µ HILIC 200 Å column (Phenomenex) as described previously (60, 61). 5-13 µM PA2602 was incubated at 37 °C with 1-21 mM L-cysteine in the presence of 20-400 µM bathocuproinedisulfonic acid (10 mM phosphate buffer, 0-20 mM NaCl, pH 7.5). Reaction was initiated by mixing 5 μ L PA2602 with 845 μ L temperate buffered substrate. The reaction mixture was incubated for 60 min with aliquots withdrawn at regular time intervals. Both substrate and product showed linear changes over 60 min and the slopes were used to calculate the respective reaction velocities. The velocities were plotted versus initial cysteine concentrations and were fitted to Michaelis-Menten equation with V_{max} and $K_{\rm m}$ constants using Prism 5 (GraphPad) software. V_{max} was normalized to the PA2602 active site iron concentration to calculate k_{cat} . Alternative reaction conditions of pH 6.5 were also tested, however the rate of reaction was slower and therefore pH 7.5 was used for subsequent kinetic experiments.

Assessment of PA2602 Substrate Specificity. p3MDO (170 μ M active site iron) cysteinespecific activity in the presence of 10 mM cysteine was tested for inhibition by 10 mM cysteine analogs (3-MPA, DL-homocysteine, N-Acetyl-L-Cysteine or cysteamine) using the HPLC-ELSD assay described above.

Quantitative assessment of 3-MPA Dioxygenase Activity of PA2602 after Fe^{II} Enrichment. A derivatization method (66) based on the reaction between the free-thiol group of 3-MPA and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, or Ellmans reagent) was adapted to quantify 3-MPA. PA2602 (9 μ M) was incubated at 37 °C with 0.2-5 mM 3-MPA (100 mM phosphate buffer, pH 7.5). Reaction was initiated by mixing 50 μ L PA2602 with 450 μ L temperate buffered substrate. Reaction aliquots were withdrawn at various time points and processed and analyzed as previously described. Michaelis-Menten parameters were obtained and analyzed in the same way as described for cysteine dioxygenation above.

Mössbauer Spectroscopy. ⁵⁷Ferrous iron stock solutions for Mössbauer spectroscopy experiments were prepared as previously described.(67) Anaerobic preparations of protein (0.8-1.5 mM) in 100 mM Tris-HCl (pH 8.0) and 50 mM NaCl were iron-saturated anaerobically by exogenously adding ferrous iron in the presence of 1 eq dithionite followed by treatment with Analytical Grade Chelex 100 sodium form, 200-400 mesh (Bio-Rad). Protein samples were frozen in liquid nitrogen in a glovebox (Belle Technology). ⁵⁷Fe Mössbauer spectra of frozen liquid samples in a custom teflon sample holder (approximately 400 µL volume) were recorded on a Mössbauer spectrometer from SEE Co. (Science Engineering & Education Co., MN) equipped with a closedcycle refrigerator system from Janis Research Co. and SHI (Sumitomo Heavy Industries Ltd.) and a temperature controller from Lakeshore Cryotronics, Inc. Data were collected in constant acceleration mode in transmission geometry with an applied field of 47 mT parallel to the y-rays. The zero velocity of the Mössbauer spectra refers to the centroid of the room temperature spectrum of a 25 µm metallic iron foil. Analyses of the spectra were conducted using the WMOSS program (SEE Co., formerly WEB Research Co. The Mössbauer spectra Edina. MN). of p3MDO:Fe^{II} resting state (n=10), cysteinesaturated (n=4) and 3-MPA-saturated (n=2)samples were recorded and average p3MDO Mössbauer parameters determined. All spectra shown in this work were fitted using a parameter file containing constrained average p3MDO Mössbauer parameters such that only relative areas of p3MDO:Fe^{II} and p3MDO:Fe^{II}:substrate species were allowed to vary.

Determination of $p3MDO:Fe^{II}:substrate$ Complex Dissociation Constants. Formation of ES complex as a function of substrate concentration was followed using Mössbauer spectroscopy and substrate bound fraction was determined assuming that the Lamb-Mössbauer factors of bound and non-bound protein were the same. The fraction bound was plotted against cysteine concentration normalized to active site ferrous iron concentration present in the sample. Consequently, a modified version of the equation describing *ES* complex formation under conditions of substrate depletion was derived: $y = 0.5(K+1+x) - (0.25(K+1+x)^2 - x)^{1/2}$, where y = [ES]/[E], x = [S]/[E] and $K = K_d/[E]$.

Synthesis of the Disodium Salt of 3sulfinopropionic Acid. 3-sulfinopropionic acid (3-SPA) was synthesized based on the method by Jollès-Bergeret(68) and a modified version by Schurmann et al.(44) All chemicals for the synthesis were used without further purification from Sigma Aldrich. Briefly an intermediate bis-(2-carboxyethyl)sulfone was formed by the condensation of acrylic acid and sodium formaldehyde sulfoxylate in aqueous solution. The intermediate precipitated was through acidification, dried and then cleaved under basic conditions. The product 3-sulfinopropionate was precipitated as a disodium salt with a large excess of an ethanol-acetone (5:1) mixture with a final vield of 61% after a second cleavage step. Elemental analysis was carried out at the Campbell Micro-analytical Laboratory, University of Otago.

Elemental analysis calcd. for C₃H₄O₄SNa₂: C = 19.8; H = 2.2; S = 17.6; Na = 25.3. Found: C = 18.5; H = 2.3; S = 12.4; Na = 23.0; m.p. 308 °C; HRMS (-ve ESI) *m/z* calcd. For [M-H]-: 136.9914, found: 136.9919; ¹H-NMR (400 MHz, D₂O): δ (ppm) 2.45 (2H, s, H2), 2.55 (2H, s, H3); ¹³C NMR (125 Mhz, D₂O): δ (ppm) 30.05 (C3), 57.23 (C2), 181.04 (C3); IR v_{max} (ATR, cm⁻¹): 2960 (C-H stretch weak), 1678 (C=O asy. stretch medium), 1420 (C=O sy. stretch strong), 1020, 1014, 975 (S=O stretch strong).

Product Determination by ¹H-NMR and ¹³C-NMR Analysis. PA2602 (200 µM) was incubated for 30 min in 10 mM phosphate buffer (pH 7.5) at 37°C in the presence of 10 mM 3-MPA. The solution was diluted 6.5 fold and measured by nuclear magnetic resonance (NMR) using a TSPd4-capillary for ¹H-NMR 500 MHz and ¹³C-NMR 125 MHz analysis at 25 °C on a Varian 500 MHz AR NMR spectrometer using VNMRJ 2.3A software. ¹H-NMR and ¹³C-NMR spectra 3-SPA dissolved in D₂O served as reference spectra for enzymatically produced 3-SPA. Considering the relatively high concentrations of reactants used in this experiment the possibility of 3-MPA autoxidation to 3-MPA disulfide was investigated. To this end, ¹H-NMR spectra of room temperature buffered solutions of 3-MPA alone, and 3-MPA

disulfide produced with H_2O_2 were measured. Using these spectra allowed estimation of 3-MPA disulfide levels in enzymatic mixtures, which represented less than one percent.

Results

Protein Sequence Identification. BlastP search(48) of putative bacterial CDO sequence (YP 299237.1) from *R. eutropha* (strain JMP134) identified twenty P. aeruginosa strains which each contained a single possible CDO homologue. The strains originated from a variety of habitats including human hosts (eg. PAO1), plant (E2), (ATCC700888) industrial water and soil (ATCC14886). The P. aeruginosa CDO homologues exhibited high sequence similarity with variations observed at only three residues: 32, 35 and 42. BlastX search (48) of P. aeruginosa PAO1 genome (NC 002516.2) using PA2602 (gi|110645304:2945264nucleotide sequence 2945869) revealed only one putative gene coding for CDO homologue. No other related gene was found. Alignment of this homologue with other CDOs including putative bacterial CDO sequence (YP 299237.1) from R. eutropha, highlighted conserved residues and location of the highly conserved cupin motif.(23) Like that of R. eutropha and other bacteria, the P. aeruginosa sequence lacks a cysteine which participates in the cysteine-tyrosine crosslink observed in all mammalian CDOs. Comparison with the previously determined CDO fingerprint motif (21) allowed unambiguous identification of all other key residues within the PA2602 sequence suggesting a possible thiol dioxygenase function (Fig 2). Purified, recombinant PA2602 protein was assessed for ligand binding and catalytic ability. As we show later it is a 3-mercaptopropionate dioxygenase and therefore name it p3MDO.

p3MDO is expressed in low levels in P. aeruginosa. To investigate whether p3MDO is expressed by *P. aeruginosa* we initially assayed thiol dioxygenase activity in cell lysate. Selected growth conditions were tried, including media containing disulfides as the sole carbon source. Under all growth conditions tried, no activity was detected. It was therefore decided to use mass spectrometry to determine whether the protein itself is expressed. *P. aeruginosa* was grown in rich media, lysed and protein separated by SDS-PAGE before analysis by mass spectrometry.

Untargeted shotgun tandem mass spectrometry failed to identify peptides of p3MDO suggesting that the protein is either absent or only present at very low abundance (data not shown). To further assess this we performed PRM assays on 7 predicted proteotypic p3MDO peptides as a more sensitive targeted approach. PRM in conjunction with a Mascot sequence database search identified 5 p3MDO peptide sequences out of the 7 recorded peptide spectra with ion scores between 36 and 85 (Fig 4). The identification is highly significant and specific for p3MDO within the 48 million sequence entries in the non-redundant NCBI sequence database. Fig 4 shows the raw fragmention spectrum of the doubly charged precursor at m/z 648.8433 and the Mascot fragment-ion assignment for the sequence LEPGEVEALSPR as a representative example of a significant peptide identification. One of the 7 fragment-ion spectra was assigned to isoprenoid biosynthesis protein from *P. aeruginosa* (gi|489228560) indicating that a false positive signal and retention time had been considered for the scheduled PRM assay and another peptide did not match any sequence in the database. The PRM assay clearly confirmed the presence of p3MDO. However, the failure to identify the protein by shotgun LC-MS/MS and the requirement of a highly sensitive targeted approach suggests that the p3MDO protein is present at very low abundance and this would explain the lack of activity observed.

Crystallographic model. Initial crystallization of p3MDO was achieved from ammonium sulfate at pH = 7.5 (crystal A) and a 2.7 Å resolution structure obtained. Crystallization using sodium acetate conditions similar to those used to crystallize rat CDO (56) produced crystals with a different space group and which diffracted to higher resolutions, 2.5 Å (crystal B) and 2.1 Å (crystal C). Data were complete and moderately redundant, with acceptable merging statistics (Table 2). Refinement yielded good geometry and good agreement with data. The four independent copies in the asymmetric unit are similar (pairwise RMSD of 0.24 to 0.40 Å) and similar to the two copies in our preliminary 3USS structure (pairwise RMSD of 0.27 to 0.44 Å, crystal A versus either crystal B or C). All monomers in both crystals participate in similar dimers, mediated by a βstrand interaction between L189 across the interface and hydrogen bonds between the side

chains of D68 and S69 on opposite molecules. These interactions may influence the orientation of these loops in these crystals. We know of no evidence that the dimer exists under physiological conditions or has biological significance.

Initially, the structure can be compared with that from R. eutropha, with which it shares high identity (62%), and which has been suggested to be a 3MDO.(69) The structures are indeed very similar with an overall RMSD of 0.66 Å. This similarity is also true of the active site (Fig 5A) where identical distances between the iron and the second sphere residues such as the hydroxyl of Y159 are observed (4.1 Å). The only notable difference is the absence of a dioxygen molecule trans to H142 that was modeled in the equivalent position in R. eutropha (4QMA). Although increased electron density was observed at that position, this was modeled as a sulfate in our original structure of p3MDO (crystal A) and in the original analysis of R. eutropha (2GM6). In our subsequent higher resolution structures presented here (crystals B and C), where sulfate was not present, we attempted modeling dioxygen and other molecules in solution but were unable to produce convincing fits in all four chains in the asymmetric unit. Without further supporting evidence, this is modeled conservatively as a water molecule.

Given the close similarity and lack of kinetic data for R. eutropha 3MDO comparisons of the bacterial structures offered only limited insights into the structural basis of function. A useful comparison was made with the structure of rat CDO (4KWJ) for which there is considerable crystallographic and kinetic data.(70-73) Alignment of the structures based on the backbone confirms the structure similarity suggested by sequence homology (RMSD of 0.86 Å, Fig 5B). This similarity is also exhibited by the organization of the active site with conserved residues occupying similar positions in both structures, even though p3MDO does not contain a cysteine-tyrosine crosslink observed in all mammalian CDO structures (Fig 5C). The iron, coordinated by three histidines and three waters has slightly distorted octahedral geometry (176 \pm 3° , $159 \pm 4^{\circ}$ and $175 \pm 3^{\circ}$, average angles as seen in all four chains). The sidechain of H142 occupies an unfavorable rotamer, as it does in all other CDO X-ray crystal structures, suggesting strain

induced by the iron coordination. Interestingly, dioxygen is proposed to bind iron at the site trans to this histidine.(74)

Surprisingly, superposition of the bacterial and rat CDO structures based on the full polypeptide chain places the catalytic iron in a different position in the two structures, although the relative position of iron, the hydroxyl O of Y159 and the δ-N of H157 remain constant (numbering from PA2602). This suggests that a more informative structural comparison is possible if these three centers are aligned. Superposition of these three conserved features (Fig 5D) displaces the β -strand formed by residues 153-159 demonstrating that the key elements of the bacterial active site are displaced by approximately 3 Å relative to the β barrel, if compared to the mammalian enzyme. This shift correlates with the presence or absence of a cis-peptide bond following the residue Cterminal to Y159. All structures of mammalian CDO show both the cysteine-tryrosine crosslink and a serine-proline cis-dipeptide immediately following the tyrosine. Both our structure and the R. eutropha structure (40MA) lack this sequence and the corresponding bond is in the trans conformation. The resulting shift of the mainchain would open a void, which is filled in the bacterial enzymes by the indole sidechain of W94, immediately preceding the glycine that replaces the cysteine observed in the mammalian crosslink. This alternate mainchain conformation allows the triangle formed by the catalytic iron, the hydroxyl O of Y159 and the δ -N of H157 to superimpose closely, with the hydroxyl of \$153 moved by 2.3 Å. This rotation of the active site relative to the β barrel results in Q62 in p3MDO occupying a very different position relative to corresponding residue R60 in the rat structure. R60 has been shown in CDO to interact with the carboxyl group of cysteine substrate when cysteine is bound as a chelate to the active site iron via its thiol and amine groups.(71)

Iron binding and active site titration. The relationship between exogenously added iron and iron bound to p3MDO is presented in Fig 6A. The initial slope of the fit (~1) reveals stoichiometric binding between iron and p3MDO. The data points were fitted to a quadratic equation(64) to estimate the upper limit(75) of the p3MDO:Fe^{II} dissociation constant as $3 \pm 1 \mu$ M. Currently, the only known enzymes with His₃ iron coordination with a

reported K_d are CDO, and *A. johnsonii* Diketonecleaving dioxygenase (Dke1). In both cases the K_d is ~5 μ M,(57,76) which is comparable with the estimate of the p3MDO affinity for iron presented here.

We then measured the cysteine-dependent oxygen depletion activity by following time dependent O₂ uptake in the presence of p3MDO with increasing concentrations of bound iron. The relationship between protein-bound iron and initial velocities of oxygen consumption exhibited a linear correlation with the slope of the best-fit line corresponding to $0.007 \pm 0.001 \text{ s}^{-1}$ (Fig 6B) reflecting the catalytic turnover number per active site bound iron. Activity plateaued when bound enzyme iron equaled total concentration suggesting а stoichiometric, catalytically competent relationship between p3MDO and iron.

Measurement of P. aeruginosa thiol dioxygenase activities. We tested p3MDO for cysteine dioxygenase activity using an HPLC assay, previously described.(77,78) Incubation reduced the cysteine-associated peak and produced a peak in the HPLC chromatogram with a retention time (~16 min) identical to that of CSA standards. In addition, samples analyzed by HPLC-ELSD were also analyzed by mass spectrometry (-ve ESI) and showed m/z peak of 152.0029 which compared well to the calculated profile (152.0023) and the CSA standard (152.0029).

Fidelity of cysteine utilization as a substrate for p3MDO was tested in a number of ways. Firstly, the rate of cysteine depletion and CSA formation could be followed by our HPLC based assay in the same experiment. The relative rates using 10 mM cysteine at 25 °C (0.005 s⁻¹ CSA formation versus 0.007 s^{-1} cysteine depletion, n=2) suggested ~70% conversion of substrate cysteine into CSA. Importantly, this rate of cysteine depletion mirrors O₂ uptake measured using an oxygen electrode described above (0.007 s^{-1}) . Finally, we took a Mössbauer sample (774 µM p3MDO) with 10 equivalents cysteine added to ensure all protein has substrate bound and added one equivalent of O₂ dissolved in buffer in a sealed system. The amount of CSA produced was determined by HPLC (560 \pm 20 μ M, n=9) and this also corresponds to ~70% use of dioxygen to produce CSA. Althought these ratios are dependent on enzyme concentration, pH and other experimental variables, the data obtained are similar to those for rat CDO with cysteine(72) and strongly support cysteine being a viable substrate for p3MDO, even if not its preferred substrate.

The steady state parameters for cysteine were determined and Fig 7 illustrates the dependence and saturation behavior of the rate of CSA formation as a function of cysteine substrate concentration. The summary of Michaelis-Menten kinetic parameters for p3MDO is shown in Table 3. p3MDO exhibited a K_m in the millimolar range (of cysteine) which is in accordance with previously published values for other bacterial CDOs (21). However, p3MDO had a significantly slower steady state rate of catalysis than other bacterial CDOs.(21)

The relatively slow rate of cysteine dioxygenation led us to investigate other thiols as potential inhibitors or substrates of the thiol dioxygenase activity of p3MDO. Cysteamine, homocysteine, N-acetyl-L-cysteine and 3-MPA were chosen. In our HPLC assays we detected only minor reduction of cysteine dioxygenase activity in the presence of stoichiometric concentrations of N-acetyl-L-cysteine, D,Lhomocysteine and cysteamine, $86 \pm 15\%$ (n = 6), $79 \pm 14\%$ (n = 8) and $75 \pm 19\%$ (n = 10), respectively. We previously showed that D,Lhomocysteine binds to the active site of rat CDO.(57) However, the reported extent of inhibition of mammalian CDOs by homocysteine remains ambiguous(17,25,79) and very inefficient use of homocysteine as a substrate has recently been reported.(80) Comparable inhibition by cysteamine has been reported for other bacterial CDOs.(21) N-acetyl-L-cysteine, homocysteine and cysteamine dioxygenation product was not observed either in the competition assays or when incubated with P. aeruginosa CDO alone. In contrast, incubation of P.aeruginosa p3MDO with cysteine in the presence of stoichiometric 3-MPA resulted in no detectable levels of CSA within the measured time window. However, HPLC chromatograms revealed a new peak (retention time ~6 min) which was later assigned to the presence of 3-SPA (see below), suggesting the possibility that 3-MPA not only competed effectively with cysteine for binding in the active site, but could be utilized as a substrate for dioxygenase reaction. This is in contrast to bacterial CDOs which showed only a modest

(<6%) inhibition of CSA production by 3-MPA and no enzymatic turnover.(21)

To confirm the formation of 3-SPA, we synthesized the disodium salt of 3-SPA by an established method (44,68) and compared ¹H- and ¹³C-NMR spectra of it and enzymatically produced 3-SPA. This, with reference to published spectra(44,81,82) unambiguously showed that PA2602 readily oxidizes 3-MPA. Further evidence was provided by mass spectrometry (-ve ESI) which showed a m/z peak of 136.9918 that compared well to the calculated profile (136.9914) and the 3-SPA standard (136.9919).

An Ellmans based method was optimized to monitor 3-MPA to 3-SPA conversion. Fig 7 illustrates the dependence and saturation behavior of the initial rate of 3-MPA substrate utilization as a function of its concentration. The summary of Michaelis-Menten kinetic parameters for PA2602 is shown in Table 3. Our kinetic data show that p3MDO exhibits K_m values in the millimolar range for both cysteine and 3-MPA. A 5-fold faster turnover rate of 3-MPA catalysis combined with a lower $K_{\rm m}$ results in a 40-fold higher overall efficiency of dioxygenation of 3-MPA as compared with cysteine. Therefore, in combination with the competition data presented above, we concluded that 3-MPA is the kinetically preferred substrate for p3MDO.

The efficiency of this reaction was also investigated. Time dependent ¹H NMR spectra were taken as described previously for CDO and cysteine.(77) The rate of 3-SPA appearance was seen to be ~95% of 3-MPA disappearance. This relationship was further confirmed by measuring substrate disappearance through Ellman's assay ($0.038 \pm 0.007 \text{ s}^{-1}$, n=4) and O₂ electrode ($0.041 \pm$ 0.008 s^{-1} , n=4) at 25 °C, with 5 mM 3-MPA (i.e. well above K_m). This confirms that both substrates are converted to product during this reaction with 3-MPA.

Substrate Binding to the Active Site. Mössbauer spectroscopy has been successfully applied by us to study active site iron interactions in *Rattus norvegicus* CDO.(57) Iron binding studies suggested a tight binding p3MDO:Fe^{II} complex (see above), which allowed anaerobic reconstitution of the active site with exogenously added anaerobic ferrous ⁵⁷Fe during preparation of protein samples for Mössbauer spectroscopy experiments. Consistent with previous studies on other non-heme mononuclear-iron enzymes, the Mössbauer spectrum of p3MDO:Fe^{II} exhibited minor heterogeneity which was substantially removed by adding 1 eq of dithionite, a common treatment of protein samples for Mössbauer spectroscopy. The resulting Mössbauer spectrum of the p3MDO:Fe^{II} resting state consists of a single broad quadrupole doublet (Fig 8A, *top spectrum*) with parameters ($\delta = 1.24 \text{ mm s}^{-1}$; $\Delta E_Q = 2.91 \text{ mm s}^{-1}$) typical for high-spin Fe^{II} in octahedral coordination.

Our kinetic data identified both cysteine and 3-MPA as substrates for p3MDO undergoing the dioxygenation reaction. Therefore, we used spectroscopy Mössbauer to study the thermodynamics of formation of the enzyme substrate complex at 20 °C (Fig 8). Addition of cysteine led to the gradual conversion of the single broad quadrupole doublet of the p3MDO:Fe^{II} resting state (Fig 8A, top spectrum) to a narrow symmetrical quadrupole doublet (Fig 5A, bottom spectrum) with Mössbauer parameters ($\delta = 1.11$ mm s⁻¹; $\Delta E_Q = 3.55$ mm s⁻¹) also consistent with homogeneous high-spin Fe^{II}. Subsequent experiments with resting state (n=10) and saturating cysteine concentrations (n=4) allowed unambiguous identification of the p3MDO:Fe^{II}:cysteine complex from which average Mössbauer parameters of both species were calculated (Table 4). The reduction in isomer shift and increase in quadrupole splitting observed upon anaerobic addition of cysteine to p3MDO $(\Delta \delta = -0.12 \text{ and } \Delta \Delta E_Q = +0.63 \text{ mm s}^{-1})$ are consistent with coordination of the thiol to iron.

Similar experiments were carried out with 3-MPA. Interestingly, initial spectra measured in the of dithionite presence used to remove heterogeneity as described for cysteine above, led to loss of iron from the protein (data not shown). Unable to fully explain this observation yet, we speculate that 3-MPA persulfide is formed in the presence of 3-MPA and dithionite as we described recently,(73) and this species is able to remove iron. The spectra were therefore repeated without pretreatment with dithionite (Fig. 8B) and are thus significantly broader than those shown for cysteine (Fig 8A). However, the 3-MPA bound species has the same isomer shift as the cysteine bound form suggesting that in both cases the thiol is coordinated. The change in quadrupole splitting upon binding of 3-MPA is quite different to

cysteine ($\Delta\Delta E_Q = -0.39$ mm s⁻¹) and this is attributable to a difference in overall charge of the *ES* complexes or different binding geometries or a combination of both effects.

Fitting spectra to a linear combination of spectra with average Mössbauer parameters for resting and ES complexes allowed quantitative monitoring of p3MDO:Fe^{II}:substrate complex formation (Fig 8). The proportion of substratebound species was determined directly from the spectra. Data from several titration experiments exploring different E/S ratios were plotted versus the concentration of cysteine added, normalized to active site iron present and fitted to a modified version of the quadratic equation (Fig 8C) (see Experimental Procedures section). The use of a quadratic equation is warranted due to comparable concentrations of receptor and ligand. We report a dissociation constant for the p3MDO:Fe^{II}:cysteine complex in the lower millimolar range ($K_d = 1.3 \pm$ 0.1 mM), while the p3MDO:Fe^{II}:3-MPA shows stoichiometric binding with a upper limit for K_d of 5 μ M. In the case of cysteine the concentration of p3MDO used in the experiments is approximately the same as K_d . This, and the fact the p3MDO:Fe^{II} concentration is known to 3 significant figures means that in this case K_d is quite well defined. In contrast, the stoichiometric binding of 3-MPA means that only an upper limit is defined.

Discussion

In contrast to other non-heme mononucleariron enzymes, mammalian and bacterial CDOs display a remarkably high specificity for their substrate cysteine. Mammalian ADO, bacterial mercaptosuccinate dioxygenase and bacterial 3MDO are only able to dioxygenate cysteamine, mercaptosuccinate and 3-MPA respectively.(6,83,84) Although recent work(80) has shown mouse CDO is able to oxidize other substrates, such as L-penicillamine, the coupling between dioxygen consumption and product formation is \leq 5% suggesting that these substrates interact with the enzyme active site and undergo catalysis very differently from the authentic substrate. In this work we identified and characterized a P. aeruginosa CDO homologue capable of oxidizing two related thiol-containing substrates, cysteine and 3-MPA, to their respective sulfinic acids with a high level of coupling to dioxygen consumption.

There is a high level of sequence identity (98.5%) within the twenty p3MDO homologues identified across twenty strains of P. aeruginosa, with variations occurring only outside the previously identified CDO fingerprint motif.(21,23) Genomic analysis (BlastX) identified only one copy of the gene coding for the thiol dioxygenase homologue per genome. Mass spectrometry has shown that the protein is indeed expressed by the bacterium, albeit at very low levels. Although we have not identified growth conditions where this enzyme is up-regulated we have shown that the thiol dioxygenase breakdown pathway is present in P. aeruginosa. The physiological role of p3MDO is unclear. P. aeruginosa was unable to grow with 3MPA as the sole carbon source; the bacteria were able to grow using cysteine as sole carbon source but a mutation in the p3MDO gene did not prevent growth, showing that p3MDO is not essential for thiol catabolism (data not shown). Whatever the physiological role of this enzyme, it is present in strains inhabiting a diverse spectrum of habitats ranging from human hosts to plants, industrial waters and soil indicating its biological importance.

Our structural data provides insight into active site organization in thiol dioxygenases and the role of the cysteine-tyrosine crosslink found in mammalian CDO. Interestingly, the active site geometry is preserved independent of its orientation to the polypeptide backbone: the distances between the iron-to-hydroxyl of Y159, iron-to-δ-nitrogen of H157 and hydroxyl of Y159to-δ-nitrogen of H157 are the same in the two structures (numbering from p3MDO). Remarkably, the side chain rings of Y159 and H157 exhibit almost perfect superposition in spite of the fact that p3MDO is unable to be crosslinked. The position of this tyrosine phenol likely depends not only on the presence or absence of crosslink, but also on the presence of a serineproline cis peptide bond in the mammalian enzyme or W94 in the bacterial. This suggests that interpretation of the role of the crosslink and of the functional consequences of its removal must account for this structural feature as well.

Kinetic characterization of p3MDO using both substrates, cysteine and 3-MPA, have shown that 3-MPA is the kinetically preferred substrate with a five-fold greater k_{cat} and eight-fold smaller K_{m} .

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The enzyme is able to utilize both substrates, although inhibition studies show that when both substrates are present only 3-MPA is oxidized.

Preference for 3-MPA can be rationalized to a certain extent through our structural studies of p3MDO. Previous structural investigations of rat CDO by us(73) and others(71) have shown that R60 is integral to cysteine substrate binding. This residue, which reorganizes upon substrate cysteine forming a sulfur/nitrogen chelate with the ferrous iron in the active site, forms an ionic bond with the carboxyl group of cysteine and holds the substrate in place. Driggers et al.(69) proposed that in the R. eutropha homologue, replacement of this arginine with O67, along with presence of R173 (R. eutropha numbering for both), supports 3-MPA substrate specificity. Indeed, structural comparison with p3MDO suggests that Q62 of the P. aeruginosa p3MDO assumes the role of R60 of rat CDO, or Q67 of the R. eutropha enzyme. Significantly, glutamine is shorter than arginine and Q62 is situated further away from the iron atom and thus further from the site of substrate The active site can therefore binding. accommodate a more elongated structure of 3-MPA bound by its thiol group alone, suggested by our recent structure of 3-MPA-persulfide bound to rat CDO.(73) Conversely, because of the weaker interaction of its carboxvlate with O62, cysteine binding would be expected to be destabilized relative to rat CDO, with its cationic arginine in this position. We do not observe for p3MDO residue R168 the potential clash with amino group of substrate cysteine suggested for R173 of the R. eutropha enzyme.(69) It is possible that R168 of p3MDO interacts favorably with the carboxylate of a cysteine substrate, depending on conformation of bound substrate. B. subtilis CDO resembles mammalian **CDOs** in lacking this glutamine/arginine retains pair, and the "mammalian" serine-cisproline noted above as lying between Y157 and R168, despite lacking the adjacent cysteine-tyrosine crosslink. This raises the possibility that the cis dipeptide is correlated with substrate specificity rather than crosslink occurrence.

We investigated the iron site of catalysis and substrate binding using Mössbauer spectroscopy. The resting state, p3MDO:Fe^{II}, exhibits a broad asymmetric quadrupole doublet with parameters similar to rat CDO. The broadness in the signal could be due heterogeneity in number of water molecules bound to the iron. This idea is supported by crystallographic studies presented here and previously(71,73) that show either one, two or three well-defined water molecules. Further support comes from the sharp symmetrical quadrupole doublet exhibited by p3MDO:Fe^{II}:cysteine. Cysteine binding must replace at least one of the three bound waters and the lowering of the isomer shift supports thiolate binding to the iron. The geometry of this species is very well-defined as exhibited by significant narrowing of the spectrum upon cysteine binding. In contrast, although 3-MPA also binds via its sulfur as shown by the lowering of the isomer shift, the broadness of the spectrum is intriguing in light of different dissociation constants described below.

The large differences in the spectra observed upon substrate binding allowed the proportion of cysteine-bound p3MDO to be determined as a function of substrate added in the absence of dioxygen. These conditions preclude catalysis and allow quantification of the enzyme-substrate complex poised for oxygen binding. p3MDO showed stoichiometric binding with 3-MPA suggesting a low dissociation constant. In contrast, we determined a K_d of 1.3 mM for the p3MDO:Fe^{II}:cysteine complex. This is substantially weaker than the low micromolar dissociation constants reported for ES complexes within other non-heme mononuclear-iron enzymes such as HPCD and TauD.(85,86) It is also very much weaker than rat CDO (unpublished results). This weaker binding constant can perhaps be related to Q62 occupying the position of R60 in rat CDO as discussed above.

In conclusion, dioxygenation occurs at an octahedral Fe^{II} ion in p3MDO but unlike other thiol dioxygenases, p3MDO has significant substrate specificity towards more than one substrate; 3-MPA and cysteine, the latter with forty-fold lower catalytic efficiency but significant efficiency of coupling to dioxygen consumption. The preference for 3-MPA can be explained by a proposed weaker binding of cysteine caused by a weak interaction between Q62 and the carboxylate of cysteine due to Q62 being uncharged, shorter and placed further from the iron than the equivalent arginine residue in rat CDO. Presence of R168 or lack of a cis-proline following Y157

may also favor 3-MPA binding over cysteine. Further studies to more clearly define how these

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

GNLJ directed the study. GNLJ and SMW conceived experiments and wrote the paper. EPT and GNLJ designed and analyzed the experiments in Figure 6 and 8. MF, ES and GNLJ designed and analyzed the experiments in Figure 7. EPT, MF and SMW designed and analyzed the experiments in Figure 5. TK designed and analyzed the experiments in Figure 4. LWM and ILL designed, performed and analyzed growth studies in *P. aeruginosa*. SA carried out kinetic studies to investigate efficiency of reaction. All authors reviewed the results and approved the final version of the manuscript.

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Footnotes:

[‡] These authors contributed equally.

[†] Abbreviations: CDO, cysteine dioxygenase; CSA, cysteine sulfinic acid; 3-MPA, 3-mercaptopropionic acid; 3-SPA, 3-sulfinopropionic acid, 3MDO, 3-mercaptopropionate dioxygenase; p3MDO, *P. aeruginosa* 3-mercaptopropionate dioxygenase; CoA, coenzyme A; BLAST, Basic Local Alignment Search Tool, SIB, Simple is Beautiful; PRM, parallel reaction monitoring; HILIC, hydrophobic interaction liquid chromatography.

Figure Legends:

Figure 1. Structural formula of thiol-containing compounds tested as substrates for *P. aeruginosa* thiol dioxygenase (p3MDO).

Figure 2. Alignment of the *P. aeruginosa* CDO homologue with selected family members. Numbering of *P. aeruginosa* residues is at top, extent of each sequene is indicated at left. Residues of the CDO fingerprint proposed by Stipanuk *et al.* are shown in white on black. White on grey indicates the possible divergence of the fingerprint in substrate specificity at position 62 in PA2602 (glutamine vs arginine), the difference in uncrosslinked residue at position 95 (glycine or alanine vs cysteine), and at position 168 (argenine vs various). Residues adjacent and possibly influencing the crosslink black on light grey. The conserved cupin motif residues are indicated on the bottom. PA2602 from *Pseudomonas aeruginosa* PAO1, NP_251292.1 is aligned with characterized 3-mercaptopropionic dioxygenases (3MDOs) (6) (*Variovorax paradoxus* TBEA6, ACB72254; *Ralstonia eutropha H16*, YP_841375); characterized cysteine dioxygenases (CDOs) (20,21,25) (*Homo sapiens*, NCBI protein sequence accession number BAA12873; *Rattus norvegicus*, BAA11925; *Streptomyces coelicolor* A3(2) [SCO3035], NP_627257; *Streptomyces coelicolor* A3(2) [SCO5772], NP_629897; *Bacillus subtilis*, NP_390992; *Bacillus cereus*, NP_832375) and putative CDOs (23) with a putative cysteine-tyrosine crosslink (*Vibrio splendidus* 12B01, EAP93565.1; *Chromobacterium violaceum* ATCC, NP_902852.1).

Figure 3. Purified samples of *P. aeruginosa* thiol dioxygenase (p3MDO) were loaded on a 15% (w/v) polyacrylamide gel, resolved by SDS-PAGE and detected by staining with Coomassie Blue dye. A series of p3MDO dilutions (1, 1:10, 1:20, 1:40). A 40-fold difference in protein sample loaded illustrates at least 97.5% purity of the protein preparation.

Figure 4. Significant identification of a proteotypic peptide sequence of p3MDO by PRM. A: Representative raw spectrum of fragment ions after collision induced dissociation of the doubly charged precursor at m/z 648.8433. Peaks annotated as y-ions (fragment ions carrying the C-terminus) were assigned to fragment ions of the peptide sequence LEPGEVEALSPR by the Mascot software. B: Detected sites of peptide fragmentation that confirm the sequence. Only y-ions were identified.

Figure 5. Structural comparison of p3MDO (4TLF, blue) with proposed 3MDO from *R. Eutropha* (4QMA, green) and rat CDO (4KWJ, red). Selected conserved sidechains are shown with oxygen, nitrogen, sulfur and iron atoms highlighted in red, blue, yellow and orange. Comparison with proposed 3MDO. (A) shows the close similarities between the active site of the two enzymes. Slightly larger electron density *trans* to H142 was modeled as O_2 in 3MDO but as water in p3MDO. Comparison with rat CDO (B) shows the overall cupin fold aligned on all backbone atoms. (C) displays the active site when the peptide backbones are aligned (iron-bound water molecules have been removed for clarity). (D) shows an alignment based on Fe, the hydroxyl O of Y159 and the δ -N of H157 (the iron-bound waters of only 4TLF are shown). This last alignment clearly shows the difference in position of Q62 compared to the corresponding residue of rat CDO, R60*. This difference can explain the difference in reactivity of p3MDO towards cysteine and 3-MPA. To aid comparison, * refers to the rat CDO sequence numbering.

Figure 6. Affinity and kinetic characterization of $p3MDO:Fe^{II}$ complex. Protein (70 µM) was mixed anaerobically with varying concentrations of ferrous sulfate. Unbound-iron was removed by Chelex under anaerobic conditions and the concentration of bound iron measured using the ferrozine assay. A) The protein-bound iron is plotted vs. total iron added and fitted according to the *Experimental Procedures*. B) Aliquots of 70 µM p3MDO with various amounts of iron bound were reacted with 10 mM cysteine at 25°C and O₂ uptake measured by oxygen electrode in an air tight system. The initial velocities of O₂ depletion were calculated by linear regression and plotted vs. the concentration of protein-bound iron. The best-fit line is plotted with a slope of 0.007 s⁻¹.

Figure 7. Michaelis-Menten kinetics of the dioxygenation reaction catalyzed by p3MDO using 3-MPA (open circles) and cysteine (full circles) as substrates measured at 37° C. Michaelis-Menten constants obtained through fitting (black lines) are reported in Table 2. Error bars represent standard deviation of at least n=3 data points.

Figure 8. Determination of p3MDO:Fe^{II}:cysteine complex dissociation constant. A) Mössbauer spectra of anaerobic p3MDO resting state (*top spectrum*) and upon anaerobically added increasing concentrations of cysteine (*spectra below*). Spectra represent data from two separate titration experiments. Cysteine concentrations were normalized to the concentration of the p3MDO active site ferrous iron present in the sample and are illustrated by the ratios. The spectra can be fitted to two quadrupole doublets employing average Mössbauer parameters (Table 3) for unbound p3MDO:Fe^{II} (red line) and substrate-bound p3MDO:Fe^{II}:cysteine (blue lines) complexes as described in Experimental procedures. B) Similar titration experiment using 3-MPA. The same color scheme is used to label bound and unbound species as for cysteine. C) The relative area occupied by cysteine- (full circles) and 3-MPA-bound (open circles) species is plotted vs. final thiol concentration (normalized to the active site concentration) and fitted according to the Experimental procedures.

Tables:

Table 1. Peptides identified by PRM and software assisted spectrum interpretation using Mascot.

Predicted <i>m/z</i> (charge state) ^a	Peptide sequence identified by Mascot	Score	Modification
gi/372467082 Chain A, Crystal Structure of Cysteine Dioxygenase from P. aeruginosa			
474.5772 (3+)	YQQYLLHVDSR	36	
648.8433 (2+)	LEPGEVEALSPR	85	
515.5866 (3+)	IGDVHQVSNAFSDR	40	
829.4547 (2+)	TSISIHVYGANIGAVR	48	
725.6833 (3+)	AVFSAEGEEKPFISGYSNSR	70	
gi/489228560 isoprenoid biosynthesis protein, partial [P. aeruginosa]			
755.7236 (3+)	LVTTPAYMLAQSIAEAASGINK	85	Oxidation (M)
No identification			
764.3578 (3+)	GAEYSQPYAFDAGGRPHPSGAR	-	

^a Predicted m/z (charge state) is the m/z of the strongest charge state predicted by the Skyline software that was used to build the scheduled PRM assay. Score: Mascot ion score at a significance threshold of p<0.05. An ion score >56 indicates a significant peptide identification in a search against 48 million sequence entries of the non redundant NCBI sequence database. All peptide sequences assigned to cysteine dioxygenase were unique for the identified protein.

	Crystal A ((NH ₄) ₂ SO ₄)	Crystal B (NaOAc)	Crystal C (NaOAc)
Resolution range (highest	48.9 – 2.70 Å (2.85-2.70 Å)	47.3 – 2.50 Å (2.64-2.50 Å)	47.2 – 2.14 Å (2.25-2.14 Å)
shell)			
Observed reflections	112,263 (16,607)	238,593 (31,848)	665,020 (92,078)
Unique reflections	16,270 (2,339)	31,044 (4,391)	49,221 (6,940)
Redundancy	6.9 (7.1)	7.7 (7.3)	13.5 (13.3)
Completeness	99.9% (99.7%)	99.9% (99.5%)	99.8% (98.7%)
I/σ	13.9 (4.2)	16.6 (2.8)	16.5 (7.7)
R _{merge} ^a	0.100 (0.464)	0.099 (0.664)	0.114 (0.371)
Reflections used in refinement	16,261 (1,180)	28,051 (4132)	45,220 (5,937)
R _{cryst} ^b	0.208 (0.283)	0.183 (0.312)	0.198 (0.271)
R _{free} ^c	0.283 (0.375)	0.245 (0.391)	0.249 (0.332)
Deviation from ideal bond	0.017Å	0.009Å	0.009Å
lengths			
Deviation from ideal bond	1.753°	1.258°	1.168°
angles		_	_
Average B factor	41.64 Å^2	40.91 Å ²	26.13 Å^2
Macromolecules	41.64 Å^2	41.02 Å^2	25.63 Å ²
Iron atoms	79.04 Å ²	42.95 $Å^2$	26.10 Å^2
Solvent	41.69 Å^2	37.42 Å^2	31.31 Å^2
PDB	3USS	-	4TLF

Table 2. Data collection, reduction and crystallographic refinement statistics.

 $^{a}R_{merge} = \Sigma |I_{obs} - I_{ave}| / \Sigma I_{ave}$

^b $R_{cryst} = \Sigma |F_{obs}-F_{calc}|/\Sigma F_{obs}$ computed over a working set composed of 95% of data.

^c $R_{free} = \Sigma |F_{obs} - F_{calc}| / \Sigma F_{obs}$ computed over a test set composed of 5% of data.

Table 3. Michaelis-Menten parameters of p3MDO measured at 37°C.

	3-MPA	Cysteine
$k_{\rm cat} ({\rm s}^{-1})$	0.11 ± 0.02^{a}	0.021 ± 0.003
$K_{\rm m}({\rm mM})$	1.0 ± 0.4	8 ± 3
$k_{\rm cat}/K_{\rm m} ({\rm M}^{-1} {\rm s}^{-1})$	110 ± 50	3 ± 2

^a Standard error represents the error associated with the fit to Michaelis-Menten equation.

Table 4. Mössbauer parameters of *P. aeruginosa* thiol dioxygenase measured at 5.2 K.

	$\delta (\text{mm s}^{-1})$	$\Delta E_{O} (mm s^{-1})$	$\Gamma_{\rm L} ({\rm mm \ s}^{-1})$	$\Gamma_{\rm R} ({\rm mm \ s}^{-1})$
Fe ^{II} :p3MDO	1.23 ± 0.02	2.92 ± 0.09	$0.57^{a} \pm 0.04$	0.80 ± 0.06
Fe ^{II} :p3MDO:cysteine	1.11 ± 0.01	3.6 ± 0.2	$0.39^{a} \pm 0.01$	$\Gamma_{\rm L} = \Gamma_{\rm R}$
Fe ^{II} :p3MDO:3-MPA	1.11 ± 0.02	2.5 ± 0.3	$0.90^{\rm b} \pm 0.2$	$\Gamma_{\rm L} = \Gamma_{\rm R}$

^a Spectra fitted using a Lorentzian lineshape.
^b Spectra fitted using Voigt lineshape (dithionite was not added).

^c Standard deviation represent errors in parameters calculated using data from at least four independent experiments. n=10 for Fe^{II}:CDO parameters.

Figures:



Figure 1

P.aeruginosa 3MDOs R.eutropha V.paradoxus	(57-168) (60-171) (58-169)	PQREQ ²² TLHVDSRQRFSVVSFV@GPEQIEP ⁹⁹¹ PYEQ ²⁰ HLHCDSAERFSVVSFV@GPEQREP ¹⁰¹ PYEQ ²¹ LHADPQDRYSVVSFV@GPEQREP ¹⁰¹ HMT-VW_LIGMMRESERAEGFAVE	142 AGGRPHPSGARRRLEPGEVEALSPRIGDVHQVSN-AFSDRTSISIEVHGANIGAVR AGGRPVPHGDAVRLLFGQVEAVSPTVGDIHRVNN-VHDDRVSVGIHVVGANIGAVR EDGKAMQLLGTEVLSPGDVDMVSPRLGDIHRVSN-MFDDRVSISIHVVGGNIGRIS
H.sapiens R.norvegicus S.ccelicolor3035 S.ccelicolor5772 B.subtilis B.cereus	(55-164) (55-164) (57-151) (49-162) (45-148) (57-171)	QYRYTRNLVDQGNG-KFNLMILC%GECHGSSIHDHTNSHGFLKMLQGNLKETLFAWE QYRYTRNLDQGNG-KFNLMILC%GECHGSSIHDHTDSHGFLKILQGNLKETLFDWE TRWHRRITCFG-YEWILS%VFCQGGGRHDHGPS VWTVLGFLTHETHERG GRUMMLAGPG-GSEAWLIG%PFTGGMHDHDSV AFVTAACELKENALAAF QYAYGNAIYRNNE-LEIVINHPFNKETYHDHQSI CAMVLECKLLNSIYRST LTQYARHLIYEDPLKRFEVLALV%KDGSSPUHDDJGW-VEVFSCRIMVKNFIG	DKKSNEMVKKSERVLRBÄQCAYINDSIG-LÄRVEN-ISHTEPAVSLÄLTSPPFDTC DKKSNEMIKKSERTLRBÄQCAYINDSIG-LÄRVEN-VSHTEPAVSLÄLTSPFDTC RA
Putative CDOs V.splendidus C.violaceum Conserved cupin m	(67-173) (57-163) Notif:	KETYCZORLFKNDHCEVLILSTLNGORSKITDHUNTSGOVKVLHGOATETLFETA DERYCZNRIYRDEHCELLLLCZREGORSOIHNEKGSLGOVRVIEGVATETVFEAT GH-HEG	anghifasosthfogCsvtvskDndhojisnloagdeplinlövgsplsof PSGolaar-Etrelaacslvinghldihovanloadggdivnihduspplsrm GP-GHN

Figure 2



Figure 3



Figure 4



Figure 5



Figure 6



Figure 7



Figure 8



Enzymology:

The Cysteine Dioxygenase Homologue from Pseudomonas aeruginosa is a **3-Mercaptopropionate Dioxygenase**

00 ... 0 **0000**---0 ENZYMOLOGY ø

Egor P. Tchesnokov, Matthias Fellner, Eleni Siakkou, Torsten Kleffmann, Lois W. Martin, Sekotilani Aloi, Iain L. Lamont, Sigurd M. Wilbanks and Guy N. L. Jameson J. Biol. Chem. published online August 13, 2015

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