Non-chemical proton-dependent steps prior to O₂-activation limit *Azotobacter vinelandii* 3-mercaptopropionic acid dioxygenase (MDO) catalysis[†]

Joshua K. Crowell, Sinjinee Sardar, Mohammad S. Hossain, Frank W. Foss Jr. and Brad S. Pierce *

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Department of Chemistry & Biochemistry, College of Science, The University of Texas at Arlington, Arlington, Texas 76019

*To whom correspondence should be addressed:

Brad S. Pierce
Department of Chemistry & Biochemistry
700 Planetarium Place, CPB Room 130
The University of Texas at Arlington
Arlington, Texas 76019
Phone: (817) 272-9066
Fax: (817) 272-3808
Email: bspierce@uta.edu

Graphical Abstract

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Joshua K. Crowell, Sinjinee Sardar, Mohammad S. Hossain, Frank W. Foss Jr. and Brad S. Pierce *

Key words

Non-heme mononuclear iron, thiol dioxygenase, solvent isotope effects, proton inventory, viscosity effects.

Abbreviations

CDO, cysteine dioxygenase; MDO, 3-mercaptopropionic acid dioxygenase; **3mpa**, 3-mercaptopropionic acid; **cys**, *L*-cysteine; **ca**, cysteamine (2-aminoethanethiol); **3spa**, 3-sulfinopropionic acid; **csa**, cysteine sulfinic acid; **ht**, hypotaurine; *Mm*, *Mus musculus*; *Av*, *Azotobacter vinelandii*; *Pa*, *Pseudomonas aeruginosa*; HPLC, high performance liquid chromatography; SKIE, solvent kinetic isotope effect.

Abstract

3-mercaptopropionate dioxygenase from *Azotobacter vinelandii* (Av MDO) is a non-heme mononuclear iron enzyme that catalyzes the O₂-dependent oxidation of 3-mercaptopropionate (**3mpa**) to produce 3-sulfinopropionic acid (**3spa**). With one exception, the active site residues of MDO are identical to bacterial cysteine dioxygenase (CDO). Specifically, the CDO Arg-residue (R50) is replaced by Gln (Q67) in MDO. Despite this minor active site perturbation, substrate-specificity of Av MDO is more relaxed as compared to CDO. In order to investigate the relative timing of chemical and non-chemical events in Av MDO catalysis, the pH/D-dependence of steady-state kinetic parameters (k_{cat} and k_{cat}/K_M) and viscosity effects are measured using two different substrates [**3mpa** and *L*-cysteine (**cys**)]. The pL-dependent activity of Av MDO in these reactions can be rationalized assuming a diprotic enzyme model in which three ionic forms of the enzyme are present [cationic, $E^{(z+1)}$; neutral, E^z ; and anionic, $E^{(z-1)}$]. The activities observed for each substrate appear to be dominated by electrostatic interactions within the enzymatic active site. Given the similarity between MDO and the more extensively characterized mammalian CDO, a tentative model for the role of the conserved ‘*catalytic triad*’ is proposed.

Introduction

Thiol dioxygenase enzymes utilize a mononuclear non-heme iron site to catalyze the O₂-dependent oxidation of thiol bearing substrates without the need for an external reductant or cofactor. Among this class of enzymes, the mammalian cysteine dioxygenase (CDO) is the best characterized [1-5]. Imbalances in *L*-cysteine (*cys*) metabolism have also been identified in a variety of neurological disease states (motor neuron, Parkinson and Alzheimer) [5-7]. These observations suggest a potential correlation between impaired sulfur metabolism, oxidative stress and neurodegenerative disease [8, 9]. For this reason, enzymes involved in sulfur-oxidation and transfer are increasingly being recognized as potential drug targets for development of antimicrobials, therapies for cancer and inflammatory disease [10-13].

Multiple high-resolution crystal structures have been solved highlighting the conserved Fe-coordination sphere among confirmed thiol dioxygenase enzymes. The typical non-heme mononuclear oxidase/oxygenase iron coordination sphere is comprised of two protein-derived neutral His residues and one monoanionic carboxylate ligand, provided by either an Asp or Glu residue. By contrast, the mononuclear iron site in all known thiol dioxygenase enzymes is coordinated by three protein derived histidine residues resulting in a neutral 3-His facial triad.

Structural comparison of annotated bacterial thiol dioxygenase enzymes suggests at least two subclasses of enzymes, which differ in a single outer Fe-coordination sphere residue (Arg or Asn) [14]. To illustrate, **Figure 1** shows the active site coordination for the ‘Arg-type’ bacterial cysteine dioxygenase (CDO) isolated from *Bacillus subtilis* (PDB code 4QM8) as compared to the ‘Gln-type’ enzyme cloned from *Pseudomonas aeruginosa* (PDB code 4TLF) [15]. Of note, the 3-His facial triad motif in both enzymes is conserved; however, the outer-sphere Arg-residue (R50) involved in electrostatic stabilization of the *cys* substrate is replaced by Gln (Q62). The

spatial orientations of all other conserved residues (S137, H139 and Y141) within the active site ‘*catalytic triad*’ remain invariant. The primary difference between the eukaryotic and bacterial ‘Arg-type’ CDO enzymes is a post-translational modification adjacent (3.3 Å) to the mononuclear Fe-site in which spatially adjacent Cys93 and Tyr157 residues are covalently cross linked to produce a C93-Y157 pair. This feature is unique to eukaryotic enzymes [16, 17].

Previously, both ‘Arg-’ and ‘Gln-type’ enzymes were annotated as bacterial CDO enzymes [14]. However, recent spectroscopic and kinetic investigations performed independently demonstrated that the ‘Gln-type’ class of thiol dioxygenase enzymes are more accurately designated as a 3-mercaptopropionic acid dioxygenase (MDO) [15, 18]. Remarkably, these ‘Gln-type’ enzymes have vastly relaxed substrate-specificity as compared to CDO [16]. Indeed, at saturating substrate concentration, MDO isolated from the soil bacteria *Azotobacter vinelandii* (*Av* MDO) demonstrated comparable activity (k_{cat}) toward three different thiol-bearing substrates [3-mercaptopropionic acid (**3mpa**), *L*-cysteine (*cys*) and cysteamine (*ca*)] [18]. Despite similar maximal velocities, the ‘Gln-type’ *Av* MDO exhibits a catalytic efficiency (k_{cat}/K_M) for **3mpa** nearly two orders of magnitude greater than *cys* and *ca*. Thus, **3mpa** appears to be the preferred substrate for this enzyme. Similar behavior was reported for the ‘Gln-type’ enzyme isolated from *Pseudomonas aeruginosa* (*Pa*) [15].

While the mammalian ‘Arg-type’ CDO isolated from *Mus musculus* (*Mm* CDO) has been extensively characterized, no information is available regarding the relative timing of chemical and non-chemical steps in ‘Gln-type’ MDO catalyzed reactions. Comparing the kinetic mechanisms for various thiol dioxygenase enzymes is critical to identifying common mechanistic behavior among this class of enzymes. To this end, the influence of pH, solvent isotope and viscosity effects were evaluated for *Av* MDO steady-state kinetic parameters (k_{cat} and

k_{cat}/K_M) obtained using two different substrates (*3mpa* and *cys*). Proton-inventory experiments were performed to determine the number of exchangeable protons in flight during chemical and non-chemical steps. The relative timing of diffusional steps was investigated by measuring the influence of solvent viscosity on enzyme kinetics. Collectively, these results provide greater insight into the role of conserved outer Fe-coordination sphere residues and the nature of substrate coordination to the mononuclear iron active site.

Materials and Methods

Expression and Purification of Av MDO

The isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible T7 vector for *Av MDO* (designated pRP42), was a generous gift from Professor Tim Larson (Virginia Tech, Department of Biochemistry). Sequence verification of the plasmid was performed by Sequetech (Mountain View, CA, <http://sequetech.com/>). The pRP42 vector was transformed into chemically competent BL21(DE3) *E. coli* (Novagen Cat. no. 70236-4) by heat-shock [42 °C for 45 seconds] and grown overnight at 37 °C on a lysogeny broth (LB) [19] agar plate in the presence of 100 mg/L ampicillin (Amp). The following day, a single colony was selected for growth in liquid LB (Amp) media for training on antibiotic prior to inoculation of 10-L BF-110 fermentor (New Brunswick Scientific) at 37 °C. Cell growth was followed by optical density at 600 nm (OD_{600}). Induction was initiated by addition of 1.0 g IPTG, 78 mg ferrous ammonium sulfate and 20 g casamino acids at an $OD_{600} \sim 4$. At the time of induction, the temperature of the bioreactor was decreased from 37 °C to 25 °C and agitation was set to maintain an O_2 concentration of 20% relative to air-saturated media. After 4 hours, the cells were harvested and pelleted by centrifugation (Beckman-Coulter Avanti J-E, JA 10.5 rotor) at $18,600 \times g$ for 15 min. The

resulting cell paste was stored at -80 °C.

In a typical purification, ~20 g frozen cell paste was added to 150 mL extraction buffer (20 mM HEPES, 50 mM NaCl, pH 8.0). Lysosyme, ribonuclease and deoxyribonuclease were added to the slurry for a final concentration of 10 µg/mL each and stirred slowly on ice for 30 minutes. The resulting suspension was pulse sonicated (Bronson Digital 250/450) for 15 seconds on/off at 60% amplitude for a total time of 15 minutes. The insoluble debris was removed from the cell free extract by centrifugation at 48,000 x g for 1 hour at 4 °C. The supernatant was diluted 1:1 with extraction buffer and then loaded onto a DEAE sepharose fast flow anion exchange column [7cm W x 20 cm L] (GE Life Sciences #17070901) pre-equilibrated with 20 mM HEPES, 50 mM NaCl, pH 8.0. The column was washed with three column volumes of extraction buffer prior to elution in a linear NaCl gradient (50 mM – 350 mM). Fractions (~10 mL) were collected overnight and pooled based on enzymatic activity as described elsewhere [18, 20]. SDS PAGE was also used to verify the presence of the recombinant protein (~23 kDa) within each fraction. Broad range protein molecular weight markers utilized in SDS PAGE experiments were purchased from Promega (Madison, WI) Cat. No. V8491. The pooled fractions were concentrated to approximately 5-10 mL using an Amicon stir cell equipped with an YM-10 ultrafiltration membrane. Thrombin protease (Biopharma Laboratories) was added to cleave the C-terminal His-tag from *Av* MDO. In a typical reaction, ~0.3 molar equivalents of thrombin per *Av* MDO (based on UV-visible absorbance at 280 nm) was added to batches of purified protein for overnight cleavage at 4 °C in HEPES buffer. The remaining thrombin and free (His)₆-tag was removed from *Av* MDO by size exclusion chromatography using a sephacryl S100 column. For all batches of *Av* MDO used in these experiments, spectrophotometric determination of ferrous and ferric iron content was measured using 2,4,6-tripyridyl-s-triazine (TPTZ) as

described elsewhere [18, 20]. For clarity, the concentrations reported in enzymatic assays reflect the concentration of ferrous iron within samples of Av MDO (Fe^{II}-MDO). Protein content was determined by Bio-Rad protein assay. NOTE: Although the expressed Av MDO has a C-terminal His-tag, as compared to the DEAE AX method described above, use of immobilized metal affinity chromatography (IMAC) results in substantially lower enzymatic activity and yields due to loss of iron and protein denaturation.

Enzyme assays

3-sulfinopropionic acid (**3spa**), cysteine sulfinic acid (**csa**) and hypotaaurine (**ht**) were assayed using the HPLC method described previously [18, 21, 22]. Instrumental conditions: column, Phenomenex C18 (100 mm x 4.6 mm); mobile phase, 20 mM sodium acetate, 0.6 % methanol, 1 % (v/v) heptafluorobutyric acid, pH 2.0. Analytes were detected spectrophotometrically at 218 nm. Reactions (1 mL) were prepared in a buffered solution at the desired pH to obtain a final concentration from 0.1 to 10 mM **3mpa** (0.1 - 60 mM **cys**). Each reaction was initiated by addition of Av MDO (typically 0.5 - 1.0 μM) at 20 ± 2 °C. Sample aliquots (250 μL) were removed from the reaction vial at selected time points and quenched by addition of 10 μL 1 N HCl (final pH 2.0). Prior to HPLC analysis, each sample was spin-filtered through a 0.22 μm cellulose acetate membrane (Corning, Spin-X). Product concentration was determined by comparison to calibration curves as described elsewhere [16, 18]. The rate of dioxygen consumption in activity assays was determined polarographically using a standard Clark electrode (Hansatech Instruments, Norfolk, England) in a jacketed 2.5 mL cell. Calibration of O₂-electrode is described in detail elsewhere [22, 23]. All reactions were initiated by addition of 1.0 μM Av MDO under identical buffer conditions as described for HPLC assays. Reaction

temperatures were maintained at 20 ± 2 °C by circulating water bath (ThermoFlex 900, Thermo Scientific).

Synthesis of the dianionic 3-sulfinopropionic acid

The salt of 3-sulfinopropionic acid (**3spa**) was prepared by saponification of the commercially available methyl ester [sodium 1-methyl 3-sulfinopropanoate; Sigma Aldrich 778168]. Briefly, sodium 1-methyl-3-sulfinopropanoate (100 mg, 0.57 mmol) was dissolved in 5 mL of deionized water. To this solution, LiOH (70 mg, 2.9 mmol) was added prior to overnight reflux under constant stirring on an oil bath (~110 °C). The solution was then cooled to ~4 °C, filtered and the filtrate was dried by rotary evaporation. The resulting solid was dissolved in 2 mL of cold ethanol and filtered again to remove inorganic salts. Evaporation of the ethanol filtrate gave the desired compound as a white solid (68 mg, 0.45 mmol, 79%). Both ^1H and ^{13}C $\{^1\text{H}\}$ NMR were measured to verify the identity and relative purity of the **3spa** product. ^1H NMR (500 MHz, D_2O) δ 2.49 (t, $J = 7.8$ Hz, 2H), 2.37 (t, $J = 7.8$ Hz, 2H). ^{13}C $\{^1\text{H}\}$ NMR (126 MHz, D_2O) δ 181.2, 57.3, 30.1 ppm. Additional confirmation of **3spa** standard was performed by mass spectrometry as described elsewhere [18] with instrumentation from the Shimadzu Center for Advanced Analytical Chemistry (The University of Texas Arlington).

Solvent kinetic isotope effects and proton inventory

For pH/D-profiles (collectively pL) and solvent isotope studies, the buffer components were prepared directly in D_2O and adjusted by direct addition of NaOD. Each pD value was obtained from the pH-electrode reading using the relationship $[\text{pD} = \text{pH} + 0.4]$. The composition of reaction buffers for all pL-profile experiments consisted of 20 mM Good's buffer and 50 mM NaCl. 2-(*N*-morpholino)ethanesulfonic acid (MES) was used to buffer reactions over the pL range of 5.5 - 6.9, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) was used to

buffer reactions over the pH/D range of 7.0 - 8.4 and 2-(cyclohexylamino)-ethanesulfonic acid (CHES) was used to buffer reactions over the pL range of 8.5 - 10. For proton inventory experiments, the mole fraction of D₂O (n) was calculated based on combining appropriate ratios of buffer prepared in D₂O and H₂O.

Viscosity studies

For solvent viscosity studies the steady-state kinetic parameters (k_{cat} and k_{cat}/K_M) were determined for Av MDO catalysis using both oxygen electrode and HPLC at pH 8.0 (20 °C) as described above. Sucrose was used to increase the buffer viscosity within reaction mixtures. The viscosity (η) of buffers containing sucrose were measured using an Ostwald viscometer relative to 20 mM HEPES, 50 mM NaCl, pH 8.2 (20 °C). The values obtained represent the average of triplicate measurements. In these experiments, addition of sucrose up to 35% (w/v) was used to increase the relative viscosity of the buffer (η_{rel}) up to ~3-times that of the control buffer.

Data Analysis

Steady-state kinetic parameters were determined by fitting data to the Michaelis-Menten equation using the program SigmaPlot ver. 11.0 (Systat Software Inc., Chicago, IL). From this analysis, both the kinetic parameters (k_{cat} and K_M) and error associated with each value were obtained by non-linear regression. For reactions where k_{cat} - or k_{cat}/K_M -pH data exhibits limiting nonzero plateaus at low (Y_L) and high (Y_H) pH, followed by transition region and subsequent decrease beyond Y_H , the results were fit to equation (1) [24]. Here, Y is defined by either k_{cat} or k_{cat}/K_M , and the variables $[H]$, K_1 and K_2 represent the hydrogen ion concentration and the two observable dissociation constants for ionizable groups involved in catalysis, respectively.

$$\text{Equation 1.} \quad \log Y = \log \left(\frac{Y_L + Y_H \frac{K_1}{[H]}}{1 + \frac{K_1}{[H]} + \frac{K_1 K_2}{[H]^2}} \right)$$

For results in which k_{cat} - or k_{cat}/K_M -pH profiles decreased only at low pH, the data were fit to equations (2) [25-27]. This expression is scaled by a constant scalar quantity (C) which represents the maximum kinetic rate (k_{cat} or k_{cat}/K_M). The error associated with each parameter (k_{cat} , k_{cat}/K_M , pK_{a1} and pK_{a2}) determined from fits to equations 1 and 2 is reported in **Table 1**.

$$\text{Equation 2.} \quad \log Y = \log \left(\frac{C}{1 + \frac{[H]}{K_1}} \right)$$

Proton inventory results were fit to equation (3) where n is the mol fraction of D_2O in the reaction chamber and E_0 and E_n are the kinetic parameters (either k_{cat} or k_{cat}/K_M) in H_2O and the mol fraction of D_2O , respectively. The measured solvent kinetic isotope effect is defined as SKIE and the integer value y describes the number of protons that contribute to the isotope effect.

$$\text{Equation 3.} \quad \frac{E_n}{E_0} = \left(1 - n + \left(\frac{n}{SKIE} \right) \right)^y$$

The effect of solvent viscosity on the steady-state parameters were fit using equation (4). In this equation, Y_0 represents each kinetic parameter determined in the absence of viscogen whereas Y_η is the value obtained at each specific relative viscosity measured. All viscosities

measured are normalized to reaction buffer in the absence of viscogen (η_{rel}). The slope of the line (m) represents the extent of diffusion limitation.

$$\text{Equation 4.} \quad \frac{Y_0}{Y_\eta} = 1 + m \cdot \eta_{rel}$$

Results

1. Solvent kinetic isotope effects

It has been previously observed that *Av MDO* catalyzes the O₂-dependent oxidation of thiol-bearing substrates 3-mercaptopropionic acid (**3mpa**), *L*-cysteine (**cys**) and cysteamine (**ca**) to yield the corresponding sulfinic acid products 3-sulfinopropionic acid (**3spa**), cysteine sulfinic acid (**csa**) and hypotaaurine (**ht**), respectively [18]. In order to probe the rate-limiting chemical and non-chemical steps in *Av MDO* catalysis, the pH/D-dependent solvent kinetic isotope effects (SKIE) for these reactions were interrogated. However, before these experiments can be presented, it is necessary to discuss some fundamental control experiments first.

For dioxygenase reactions, oxygen is a co-substrate and thus it is important to verify that atmospheric O₂-concentration is sufficient to saturate enzyme kinetics for all substrates utilized in solvent isotope experiments. This control was performed previously at pH 7.5 for all substrates (**3mpa**, **cys** and **ca**) [18]. As an addition control for these studies, this was repeated at pH 5.5, 7.5 and 10 for both **3mpa** and **cys** to verify that pH does not influence O₂-saturation. As before, steady-state rates for *Av MDO* at saturating substrate concentration are independent of oxygen within the range of 25-400 μ M. These observations indicate that (regardless of pH) the apparent $K_M^{O_2}$ (for both substrates) must be substantially lower ($\sim 10\times$) than the lowest value of oxygen used (25 μ M) [21, 28]. Therefore, any differences observed in *Av MDO* reactivity

cannot be attributed to incomplete O₂-saturation. This also means that under these conditions, the reaction of the substrate-bound Av MDO with oxygen can be considered irreversible.

An additional factor to consider is the ‘*coupling efficiency*’ of the enzyme. The efficiency at which an oxygenase enzyme incorporates one mol of O₂ into the product is commonly referred to as ‘*coupling*’. As k_{cat} represents the zero-order limit of catalysis, the coupling efficiency for a dioxygenase can be obtained from the ratio of the k_{cat} as measured from product formation divided by the k_{cat} obtained from O₂-consumption. It was previously reported that Av MDO reactions using either **3mpa** ($102 \pm 8\%$) or **cys** ($97 \pm 6\%$) as a substrate were nearly fully coupled over the accessible pH range of the enzyme [$6 < \text{pH} < 9$] [18]. By contrast, reactions utilizing **ca** as a substrate exhibited significantly lower coupling ($40 \pm 9\%$). Regardless of substrate, the coupling efficiency of Av MDO was not influenced by D₂O. Uncoupled reactions can result in the promiscuous release of reactive oxygen species such as H₂O₂, O₂^{•-} and [•]OH, which can complicate interpretation of these experiments. For this reason, the pH/D-dependent solvent isotope studies are focused on Av MDO substrates **3mpa** and **cys**, which exhibit stoichiometric coupling.

Insight into ionizable groups involved in catalysis can be obtained by measuring solvent isotope effects on the pH/D-dependence of both k_{cat} and k_{cat}/K_M . The influence of pH on Av MDO catalysis was measured over the accessible pH range of the enzyme ($6 < \text{pH} < 9$). **Figure 2** illustrates the $\log(k_{cat})$ -pH (**A**) and $\log(k_{cat}/K_M)$ -pH profiles (**B**) obtained from the initial rate data of Av MDO reactions using **3mpa** (H₂O, *white circle*) as a substrate. For comparison the $\log(k_{cat})$ -pD and $\log(k_{cat}/K_M)$ -pD profiles obtained for **3mpa** reactions are also included (*black circles*). Each point within these data sets was obtained by fitting the steady-state kinetic results observed at a fixed pL-value to the standard Michaelis-Menten equation. The error in each

kinetic parameter obtained (k_{cat} and k_{cat}/K_M) from these fits is indicated graphically using error bars.

In reactions using **3mpa** as substrate, both pH-profiles [k_{cat} -pH and k_{cat}/K_M -pH] exhibit a skewed ‘bell shaped’ curve with two apparent pKa values. Within the acidic branch of this profile [$6 < \text{pH} < 8$], both data sets exhibit sigmoidal behavior bracketed by limiting nonzero plateaus at low and high pH. Following a short transition, k_{cat} and k_{cat}/K_M values decrease beyond with a unitary slope. Over the entire pH range utilized in these experiments, both data sets (k_{cat} and k_{cat}/K_M) can be reasonably fit to equation 1 (*solid line*). The limiting values of k_{cat} spans $0.17 \pm 0.01 \text{ s}^{-1}$ (Y_L) to $0.45 \pm 0.06 \text{ s}^{-1}$ (Y_H) at 20 °C. Likewise, the limiting values for k_{cat}/K_M within the sigmoidal region lie between $10,000 \pm 1,100 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $27,000 \pm 3,000 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively. Relative to H₂O reactions, the observed k_{cat} values are unaffected for *Av* MDO assays carried out in D₂O (**Fig. 2, A**). By contrast, a significant decrease in k_{cat}/K_M is observed for **3mpa** reactions carried out in D₂O (**Fig. 2, B**). The sigmoidal limits of k_{cat}/K_M -values in D₂O lie between $3,200 \pm 200 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $9,600 \pm 1,200 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively.

For each pH-profile, the two ionizable groups (pKa₁ and pKa₂) were determined by fitting data to equation 1. From this analysis, the pKa values (pKa₁, 7.8 ± 0.2) and (pKa₂, 9.2 ± 0.1) were obtained from the **3mpa** k_{cat} -pH profile in H₂O. Similarly, two pKa values (pKa₁, 7.4 ± 0.2) and (pKa₂, 9.1 ± 0.1) were obtained from the **3mpa** k_{cat}/K_M -pH profile. As with H₂O reactions, two ionizable groups are also observed in *Av* MDO D₂O assays using **3mpa** as a substrate. In fact, the log(k_{cat})-pD profile obtained is essentially super imposable with the results obtained in H₂O. Thus, within experimental error, the presence of D₂O has no influence on the pKa-values obtained from k_{cat} data. By contrast, the value of pKa₁ obtained from log(k_{cat}/K_M)-pD fits (7.8 ± 0.2) is shifted more basic by $\Delta \text{pKa}_1 = + 0.4$, whereas the value of pKa₂ (9.2 ± 0.1)

remains largely unperturbed. The increased in pK_{a1} is consistent with ionizable groups involved with catalysis [29, 30].

The pL-profile for Av MDO catalyzed reactions utilizing *cys* as substrate (**Fig. 2, C and D**) exhibits a ‘bell shaped’ curve suggesting two ionizable groups. However, the second pK_a -value is predicted to fall outside of the pH range of the experimental data set (> 10). Therefore, k_{cat} - and k_{cat}/K_M results obtained in either H_2O or D_2O were fit to equation 2 to obtain pK_{a1} . In this analysis, data collected beyond pH 9 was neglected. In *cys* reactions carried out in H_2O , a pK_a value of 6.3 ± 0.1 was obtained from $\log(k_{cat})$ -pH fits. As with *3mpa* reactions, no significant perturbation is observed in the value of k_{cat} in H_2O ($0.28 \pm 0.02 \text{ s}^{-1}$) as compared to that obtained in D_2O ($0.26 \pm 0.02 \text{ s}^{-1}$). However, the observed pK_a -value is shifted up to 7.1 ± 0.1 ($\Delta pK_{a1} = +0.8$) in D_2O . Similarly, the observed pK_a -value (pK_{a1}) obtained from $\log(k_{cat}/K_M)$ -pL fits of *cys* reactions in H_2O are shifted upward to from 6.2 ± 0.1 to 7.2 ± 0.1 ($\Delta pK_{a1} = +1.0$) in D_2O . Regardless, no solvent isotope effect is observed on either k_{cat} or k_{cat}/K_M for Av MDO catalyzed reactions with *cys*. A summary of the all pH/D-dependent kinetic results is provided in **Table 1**.

For each substrate utilized (*3mpa* and *cys*), the solvent kinetic isotope effect (SKIE) on each steady-state kinetic parameters (k_{cat} and k_{cat}/K_M) was determined within the pH/D-independent region of each pL-profile. For example, the k_{cat}/K_M -pL-profile for *3mpa* (**Fig. 2B**) exhibits two pH-independent regions (pL ~ 6.0 and 8.2), both of which exhibit a solvent kinetic isotope effect of 2.7 ± 0.4 . This value is significantly larger than possible if solely attributed to differences in the relative viscosity of a D_2O solution as compared to H_2O . Nevertheless, additional viscosity experiments are presented below to determine if the attenuation of the k_{cat}/K_M -values obtained with *3mpa* in D_2O has contributions from viscosity effects. Alternatively, as illustrated in **Fig. 2C and D**, neither k_{cat} -pL nor k_{cat}/K_M -pL profiles for Av MDO catalyzed *cys* reactions exhibit

solvent isotope effects beyond experimental error. Similarly, while the full pD-dependence was not determined for Av MDO catalyzed **ca** reactions; solvent isotope effects were not observed for either k_{cat} or k_{cat}/K_M in assays using **ca** as a substrate at pL 6.0 or 8.2.

2. Proton Inventories

Proton inventory experiments were performed to provide greater insight into the observed solvent isotope effect on k_{cat}/K_M for Av MDO catalyzed **3mpa** reactions. In these experiments, both steady-state kinetic parameters (k_{cat} and k_{cat}/K_M) were determined for various molar ratios of H₂O and D₂O at both pH/D-independent points (pL ~ 6.0 and 8.2). For each mole fraction of D₂O measured, the value of k_{cat}/K_M observed ($[V/K]_n$) is normalized for the value obtained in pure H₂O ($[V/K]_0$). As illustrated in **Figure 3**, the relative change in k_{cat}/K_M ($[V/K]_0/[V/K]_n$) decreases with increasing D₂O mole fraction. Proton inventory experiments performed at pL 6.0 (*black triangles*) and pL 8.2 (*white triangles*) exhibit nearly equivalent behavior indicating that there is no change in the number of rate limiting protonation steps at either the low or high branch of the pL-profile. Assuming a single proton in flight (*solid line*, $y = 1$), the best fit the observed proton inventory data to equation **3** was obtained for a solvent isotope effect of 2.63 ± 0.68 ($R^2 = 0.96_6$). Within error, this is in good agreement with the value determined from the k_{cat}/K_M -pL profile (2.7 ± 0.4). This observation suggests a single solvent exchangeable proton is in flight prior to the first irreversible step. Further, the equivalence of proton inventories within each pL-independent limb (6.0 and 8.2) implies that the ionizable group associated with pKa₁ is not the source of the rate-limiting proton.

3. Viscosity effects

The possibility that diffusion controlled events such as substrate and/or product movement into and out of the active site limit the rate of catalysis was evaluated by measuring the

perturbation of steady-state parameters (k_{cat} and k_{cat}/K_M) as a function of solvent viscosity. Measurements were made at pH 8.2 as described in *Material and Methods*. For each assay, sucrose was added to increase the relative viscosity of the reaction buffer and the results were fit to equation 4. **Figure 4** illustrates the relative perturbation to k_{cat} (or k_{cat}/K_M) obtained for reactions in the absence of viscosigen (k_0) normalized for the values obtained at selected relative viscosities (k_n). Assays using **3mpa** and **cys** as a substrate for Av MDO are shown in **Fig. 4** panels **A** and **B**, respectively. The dashed lines represent the theoretical limits of this experiment. A positive slope of 1.0 is expected if catalysis is fully diffusion-limited by non-chemical steps in catalysis. Alternatively, the horizontal line represents the expected result in the absence of any diffusional limitation [31]. The absence of any appreciable influence of solvent viscosity on k_{cat} or k_{cat}/K_M for either substrate (**3mpa** or **cys**) clearly demonstrates that Av MDO catalysis is not limited by either substrate binding or product release. Moreover, this result verifies that the increased viscosity of D₂O buffers does not contribute to the observed SKIE for Av MDO k_{cat}/K_M data obtained in **3mpa** reactions.

Discussion

Among thiol dioxygenase enzymes, the mammalian ‘Arg-type’ CDO has been extensively characterized spectroscopically [32-35], crystallographically [36-38] and kinetically [16, 22, 23]. However, almost no information is available regarding the relative timing of chemical and non-chemical steps in ‘Gln-type’ MDO catalyzed reactions. A significant point of divergence between CDO and MDO is with respect to substrate coordination to the mononuclear Fe-site. EPR spectroscopic studies indicate that substrate coordination to the Av MDO Fe-site is via thiolate-only [18]. By contrast, all known ‘Arg-type’ CDO enzymes bind **cys** via bidentate

coordination of substrate-thiolate and neutral amine. This difference in Fe-coordination likely explains the relaxed substrate specificity reported among ‘Gln-type’ thiol dioxygenase enzymes [15, 18]. Therefore, this subset of enzymes offers a unique point of comparison to better understand the significance of the first- and outer Fe-coordination sphere on catalysis and substrate-specificity among thiol dioxygenase enzymes.

For diffusion-controlled reactions in which product release is rate-limiting, k_{cat} would be attenuated by increased solvent viscosity. Since addition of D₂O to reaction buffers increases the relative viscosity of reaction mixtures, it is possible that viscosity effects contribute to the apparent solvent isotope effect. However, the results shown in **Fig. 4** clearly demonstrate that, regardless of substrate, solvent viscosity has no impact on either k_{cat} or k_{cat}/K_M . These experiments verify that non-chemical diffusional steps (*product release* and/or *substrate binding*) are not rate-limiting for Av MDO catalyzed reactions with **3mpa** or *cys*. By contrast, product release in *Mm* CDO is partially rate-limiting [21]. However, substrate (*and potentially product*) coordination for CDO is bidentate, the resulting chelate effect likely stabilizes both the **ES**- and **EP**-complex. By contrast, substrate binding to the Av MDO Fe-site is believed to be via thiolate only, and thus product release is expected to be faster by comparison.

Within the experimental pH range of $6 < \text{pH} < 8.5$, both k_{cat} - and k_{cat}/K_M -pH profiles for Av MDO reactions using **3mpa** as a substrate exhibit nonzero limiting values. Only at pH values beyond ~ 9 does the activity of Av MDO decrease linearly toward zero. By contrast, both k_{cat} -pH profiles for enzymatic reactions using substrates bearing an amino functional group (*cys* and *ca*) decrease to zero within the acidic limb of the profile [18]. These observations are key to the development of a MDO kinetic model.

The pH-dependent behavior observed in *3mpa* reactions can be rationalized assuming a diprotic model in which three ionic forms of the enzyme are present but only two of them are catalytically active [39, 40]. As illustrated in **Scheme 1**, three ionic forms of the enzyme are produced by either protonation of the neutral enzyme (designated E^Z) to form the cationic $E^{(Z+1)}$ enzyme or deprotonation to yield the anionic $E^{(Z-1)}$ form. The pH-dependent profile for *3mpa* reactions suggest that both E^Z and $E^{(Z+1)}$ are catalytically active with rates reflected by the asymptotic limits of k_{cat} within the acidic branch of the pH-profile (27,000 and 10,000 $M^{-1}s^{-1}$, respectively). However, the doubly deprotonated enzyme is either unable to bind *3mpa* or cannot reductively activate oxygen to generate product. Either way, the $E^{(Z-1)}$ ionic form is catalytically inactive in *3mpa* assays.

In the context of the reaction scheme proposed, the observed pKa values in k_{cat} - [pKa₁ = 7.4 ± 0.2; pKa₂ = 9.1 ± 0.1] and k_{cat}/K_M -pH profiles [pKa₁ = 7.8 ± 0.2; pKa₂ = 9.2 ± 0.1] are attributed to catalytically essential ionizable amino acids within the free enzyme [27]. Further, the significant solvent isotope effect (2.7 ± 0.4) observed for *3mpa* k_{cat}/K_M data suggests that proton-dependent steps play an important role in the initial reversible steps leading up to O₂ binding.

Unambiguous identification of the specific ionizing amino acid residue associated with each pKa-value is not possible without direct comparison to *Av* MDO active site variants. While such experiments are currently ongoing, a reasonable mechanistic hypothesis can be formulated based on: (1) the pKa-values observed in k_{cat} - and k_{cat}/K_M -data, (2) steady-state results obtained previously for H155A and Y157F *Mm* CDO variants, (3) and computationally validated EPR and CD/MCD studies [41, 42].

The crystal structure for *Av* MDO is not available; however, structural comparisons can be made to the *Pseudomonas aeruginosa* ‘Gln-type’ MDO (PDB code 4TLF) which shares 69.5% sequence identity (84.8% *similarity*) with *Av* MDO [15]. As noted previously, the active site of *Mm* CDO is essentially equivalent to MDO with the notable exception of the C93-Y157 pair and R60 (replaced by Q62 in MDO). The pKa-values observed in *3mpa* k_{cat} - and k_{cat}/K_M -pL profiles (7.4 - 7.8), along with the ΔpK_a (+0.4) observed in D₂O reactions, is consistent with values reported for catalytically essential histidine residues [29, 43]. As illustrated in **Fig. 1**, all known thiol dioxygenase enzymes have four conserved histidine residues within the active site. Three of which compose the Fe-coordination site (H89, H91 and H142 for *Pa* MDO, **Fig. 1B**). Protonation of any of these residues would result in loss of iron and inactivation of the enzyme. Since activity is not abolished below pKa₁ in *3mpa* reactions, it can be concluded that these residues are not protonated. This leaves only the conserved His-residue (H157) for further consideration.

The hypothesis that pKa₁ corresponds to H157 is attractive since it has been demonstrated previously that the equivalent residue in *Mm* CDO (H155) is critical for enzymatic activity. Indeed, despite comparable ferrous iron incorporation, the specific activity of the H155A *Mm* CDO variant is decreased by ~ 2-orders of magnitude relative to the wild-type enzyme [22]. Furthermore, recent spectroscopic (CD/MCD), crystallographic and computational studies of the *Mm* CDO H155A variant suggests that H155 serves as a hydrogen bond donor for the Y157 phenol group, thereby positioning the Y157 hydroxyl group of the C93-Y157 pair optimally to donate a hydrogen bond to the Fe-bound *cys*-carboxylate [42]. Assuming a similar function in the bacterial enzyme, the conserved H157 residue in *Pa* MDO would be expected to donate a hydrogen bond to the O-atom of the Y159 hydroxyl group to promote interaction with the

substrate-carboxylate. This hypothesis presents an attractive explanation for the role of the ‘*catalytic triad*’ of Ser-His-Tyr residues universally conserved among thiol dioxygenases [44]. In this model, the hydroxyl group of S155 donates a hydrogen bond to the N_ε-atom of H157 to align the opposite N_δ-atom for hydrogen bond donation to the O-atom of the Y159 phenol group. Protonation of H159 would disrupt this hydrogen bond network and result in formation of a positive charge within the enzymatic active site pocket resulting in attenuation of enzymatic activity below pK_{a1}.

By extension, the basic pK_{a2}-value observed in *3mpa* reactions is close to values typically associated with tyrosine residues (pK_a ~ 10). Furthermore, despite stabilizing a ferrous iron resting state, the Y157F (equivalent to Y159 of *Pa* MDO) variant of *Mm* CDO is catalytically inactive, thereby establishing that this residue is also essential for native catalysis [22]. Computational studies validated by EPR spectroscopy confirm that the hydroxyl-group of Y157 within the *Mm* CDO active site directly interacts with the carboxylate-group of the Fe-bound substrate (*cys*) [22]. It is therefore reasonable to expect that Y159 of MDO is the second ionizable group observed in *3mpa* pL-profiles. A schematic representation summarizing the proposed substrate-bound ionic enzymes forms [**E^(z+1)S**, **E^zS** and **E^(z-1)S**] is shown in **Scheme 2**.

Since both pK_a-values are observed in *k_{cat}*- and *k_{cat}/K_M*-pH profiles, it can also be argued that a proton-dependent step is involved in the chemical steps [27]. However, this can also be rationalized in the context of the proposed ionic enzyme forms produced as a function of pH.

As with most non-heme oxidase/oxygenase enzymes, binding and reductive activation of molecular oxygen is gated by substrate coordination to the Fe-site [18]. While the precise mechanism remains a matter of considerable debate, it has been proposed that the coordination of substrate to the Fe-site alters the Fe^{II}/Fe^{III} redox couple and/or induces key conformational

changes that facilitate direct O₂-coordination and activation [45-47]. Thus, charge stabilization and geometry of the substrate-bound Fe-site are critical for enzymatic activity.

In the doubly deprotonated $E^{(z-1)}$ form, electrostatic repulsion between the Y159-tyrosinate and *3mpa*-carboxylate group would significantly inhibit substrate binding to the opposite face of the 3-His facial triad. For perspective, the distance separating Y159 O-atom and the Fe-bound solvent **W3** is 2.67 Å [Fig. 1B]. The short distance separating two negative charges would produce a significant columbic force, likely resulting in both geometric and electrostatic distortions at the substrate-bound Fe-site. Whether the anionic $E^{(z-1)}$ form cannot bind *3mpa* or the $E^{(z-1)}$ S complex is unable to activate oxygen; the result is the same, abolishment of enzymatic activity beyond pK_{a2}. This hypothesis also provides an explanation for the absence of a basic pK_a in *k_{cat}*-pH profiles obtained in assays using *ca* as a substrate [18]. Unlike *3mpa* and *cys*, cysteamine lacks a carboxylate group. Since there is no electrostatic repulsion between the substrate and the anionic $E^{(z-1)}$ enzyme, neither substrate-binding nor subsequent O₂-activation are attenuated under basic conditions. Furthermore, the retention of $E^{(z-1)}$ activity in reactions utilizing *ca* demonstrates that the second protonation step does not irreversibly inactivate MDO.

Similarly, the differential pH-dependent behavior exhibited within the acidic limb of *3mpa* reactions as compared to substrates bearing a positively charged quaternary amine (*cys* and *ca*) can also be explained by this kinetic mechanism. As noted previously, both *k_{cat}* and *k_{cat}*/*K_M* decrease for Av MDO catalyzed *3mpa* reactions, but activity is not abolished within the acidic limb of the pH profile. Conversely, steady-state parameters approach zero for *cys* and *ca* enzymatic reactions below pK_{a1}. Protonation of H157 would produce a cationic $E^{(z+1)}$ enzyme form. The positive charged imidazole ring of H157 is positioned along the substrate-binding face of the 3-His facial triad (3.90 Å distance from the Fe-bound solvent molecule **W3**).

Therefore, cationic substrates such as *cys* and *ca* would experience considerable electrostatic repulsion under acidic conditions. Finally, the zwitterionic *cys* substrate has both amine- and carboxylate-groups, thus only the neutral $\mathbf{E}^z$ enzyme form retains activity, whereas the ionic $\mathbf{E}^{(z+1)}$ and $\mathbf{E}^{(z-1)}$ forms are inactive. As expected, this results in an inverted '*bell shaped*' curve in k_{cat} -pH profiles.

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Table 1. Summary of pL-dependent steady-state kinetic parameters determined for MDO in reactions utilizing 3-mercaptopropionic acid and L-cysteine.

Av MDO kinetic parameter	Substrate			
	<i>3mpa</i>		<i>cys</i>	
	H ₂ O	D ₂ O	H ₂ O	D ₂ O
log(k_{cat})-pL				
maximum k_{cat} (s ⁻¹)	0.45 ± 0.06	0.45 ± 0.03	0.28 ± 0.02	0.26 ± 0.02
pK _{a1}	7.8 ± 0.2	7.9 ± 0.1	6.3 ± 0.1	7.1 ± 0.1
pK _{a2}	9.2 ± 0.1	9.3 ± 0.1	-	-
SKIE	1.01 ± 0.11		1.08 ± 0.10	
log(k_{cat}/K_M)-pL				
maximum k_{cat}/K_M (M ⁻¹ s ⁻¹)	27000 ± 3000	10000 ± 1100	41.5 ± 0.7	40.8 ± 0.5
pK _{a1}	7.4 ± 0.2	7.8 ± 0.2	6.2 ± 0.1	7.2 ± 0.1
pK _{a2}	9.1 ± 0.1	9.2 ± 0.1	-	-
SKIE	2.7 ± 0.4		1.02 ± 0.09	
proton inventory				
pL 6.0	y = 1		<i>n/a</i>	
pL = 8.2	y = 1		<i>n/a</i>	

Scheme 1. Proposed kinetic mechanism for *Av*MDO catalyzed *3mpa* reaction.

Scheme 2. Proposed substrate-bound ionic enzymes forms [$E^{(z+1)}S$, E^zS and $E^{(z-1)}S$].

Figure 1

Figure 1. Crystal structure of the substrate-bound ‘Arg-type’ *Bacillus subtilis* active site (**A**, PDB code 4QM8) as compared to the annotated ‘Gln-type’ MDO isolated from *Pseudomonas aeruginosa* (**B**, PDB code 4TLF) [14, 15]. Solvent waters are designated **W1-W3**. Selected distances **A**: **1** (2.59 Å); **2** (3.30 Å); **3** (2.14 Å); **4** (2.65 Å); **B**: **1'** (2.56 Å); **2'** (2.95 Å)

Figure 2.

Figure 2. pL-dependence of steady-state kinetic parameters of MDO using 3-mercaptopropionic acid (circle, **A/B**) and *L*-cysteine (square, **C/D**) as substrates. For both substrates, $\log(V)$ and $\log(V/K)$ pL-profiles were collected in H₂O (*white*) and D₂O (*black*) and fit to **eq. 1** and/or **eq. 2**.

A summary of the results obtained from these fits are summarized in **Table 1**.

Figure 3.

Figure 3. Proton inventory of MDO catalyzed formation at pL 6.0 (*black triangle*) and 8.2 (*white triangle*). Plot of normalized V/K ($[V/K]_n/[V/K]_0$) versus mole fraction D_2O (n). Experimentally determined SKIE values for k_{cat}/K_M were used to fit data to equation 3. Proton inventory data were fit assuming a single proton in flight (*solid line*, $y = 1$). Fitting results: ${}^{\text{D}_2\text{O}}V/K = 2.63 \pm 0.68$, $y = 1$, $R^2 = 0.96_6$.

Figure 4.

Figure 4. Effect of solvent viscosity on the maximal rate ($v_0/[E]$) of MDO catalyzed *3spa* and *csa* formation. **A.** The effect of solvent viscosity on k_{cat} (k_0/k_n) and k_{cat}/K_M $[(V/K)_0/(V/K)_n]$ for *3spa* formation is designated by circles (*white*) and squares (*gray*), respectively. **B.** For comparison, the effect of solvent viscosity on k_{cat} [(k_0/k_n) , *black circle*] and $[(V/K)_0/(V/K)_n]$, *gray square*) for *csa* formation was also measured as a function of viscosity. The dashed lines represent the theoretical limits for diffusion-limited product release.

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