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Steady-state substrate specificity and O_2-coupling efficiency of mouse cysteine dioxygenase ☆

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ABSTRACT

Cysteine dioxygenase (CDO) is a non-heme mononuclear iron enzyme that catalyzes the oxygen-dependent oxidation of L-cysteine (Cys) to produce L-cysteine sulfinic acid (CSA). Sequence alignment of mammalian CDO with recently discovered thiol dioxygenase enzymes suggests that the mononuclear iron site within all enzymes in this class share a common 3-His first coordination sphere. This implies a similar mechanistic paradigm among thiol dioxygenase enzymes. Although steady-state studies were first reported for mammalian CDO over 45 years ago, detailed analysis of the specificity for alternative thiol-bearing substrates and their oxidative coupling efficiencies have not been reported for this enzyme. Assuming a similar mechanistic theme among this class of enzymes, characterization of the CDO substrate specificity may provide valuable insight into substrate-active site intermolecular during thiol oxidation. In this work, the substrate-specificity for wild-type *Mus musculus* CDO was investigated using NMR spectroscopy and LC–MS for a variety of thiol-bearing substrates. Tandem mass spectrometry was used to confirm dioxygenase activity for each non-native substrate investigated. Steady-state Michaelis–Menten parameters for sulfinic acid product formation and O_2-consumption were compared to establish the coupling efficiency for each reaction. In light of these results, the minimal substrate requirements for CDO catalysis and O_2-activation are discussed.

Introduction

Cysteine dioxygenase (CDO)[1] is a mononuclear non-heme iron enzyme that catalyzes the first concerted step in the O_2-dependent oxidation of L-cysteine (Cys) to produce cysteine sulfinic acid (CSA) (Scheme 1). 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 [1–4]. Recently, the interplay between dysfunction in sulfur metabolism and human neurodegenerative disease states (Alzheimers, autism, and Parkinsons) has been of considerable medical interest [5–7].

Multiple high resolution crystal structures of the resting and substrate-bound enzyme have been solved which highlight the atypical mononuclear iron coordination for the mammalian CDO active site [6,8–10]. Among the non-heme mononuclear iron oxidase/oxygenase class of enzymes, the typical Fe-coordination sphere is comprised of two protein-derived neutral His residues and one monoanionic carboxylate ligand provided by either an Asp or Glu residue. Unlike most enzymes within this family, one face of the CDO mononuclear iron active site (Fig. 1) is coordinated by 3 protein derived histidine residues resulting in a 3-His facial triad. Another unusual feature observed within the mature eukaryotic CDO active site is a post-translational modification in which spatially adjacent Cys93 and Tyr157 residues are covalently cross-linked to produce a C93-Y157 pair. Among CDO enzymes identified, Y157 is conserved across phylogenic domains whereas the C93-Y157 pair is unique to eukaryotes. Several reports have demonstrated that formation of this cross-link increases the catalytic activity and coupling efficiency of CDO [11–13]. Regardless, C93A CDO variants, which lack the ability to produce the

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[1] *Abbreviations used:* TDO, thiol dioxygenase; CDO, cysteine dioxygenase; ADO, cysteamine dioxygenase; MSDO, mercaptosuccinate dioxygenase; MPDO, 3-mercaptopropionate dioxygenase; Cys, cysteine; Hcy, homocysteine; CA, 2-aminoethanethiol (*cysteamine*); MS, mercaptosuccinate; PA, L-penicillamine, CME, L-cysteine methyl ester; DME, 2-(dimethylamino)ethanethiol; SC, S-methyl-L-cysteine; 3MP, 3-mercaptopropionate; 1MP; 1-mercaptopropane; HT, hypotaurine; TMSP, trimethylsilyl propanoic acid; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; LC–MS, liquid chromatography–mass spectrometry; MRM, multiple reaction monitoring; SIM, single ion mode.

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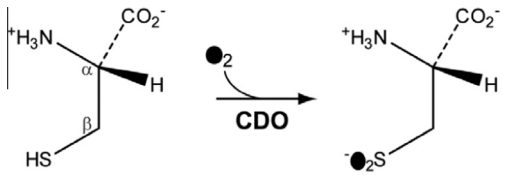
C93-Y157 pair, retain their catalytic activity. Therefore, this post-translational modification does not appear to be required for catalysis. The exact mechanism of C93-Y157 formation remains unresolved.

As shown in Fig. 1, the L-Cys substrate coordinates to the Fe-site in a bidentate manner via neutral amine and thiolate functional groups [10]. Like other members of the non-heme mononuclear iron enzyme family, CDO exhibits an obligate-ordered binding of the L-Cys substrate prior to molecular oxygen [14,15].

Cysteine dioxygenase and cysteamine (2-aminoethanethiol) dioxygenase (ADO) are the only known mammalian thiol dioxygenase (TDO) enzymes. Until recently, the catabolic dissimilation of L-cysteine was believed to be unique within the domain of eukaryotes [16,17]. However, a number of bacterial TDO enzymes have now been identified, suggesting that the ability to oxidize excess thiols is advantageous for survival. For example, two Fe/O_2-dependent TDO enzymes isolated from *Variovorax paradoxus* have recently been identified [mercaptosuccinate dioxygenase, (MSDO) and 3-mercaptopropionate dioxygenase (MPDO)] [18,19]. Sequence comparison suggests these enzymes also contain a 3-His active site motif. The conserved metal binding site among TDO enzymes suggest that the first-coordination sphere is necessary for thiol oxidation whereas the outer-sphere residues most likely facilitate binding of their specific substrates. This implies that TDO enzymes have the potential to catalyze the O_2-dependent oxidation of a variety of thiol-substrates to produce the corresponding sulfinic acid providing they are capable of binding to the mononuclear active site.

All TDO enzymes identified belong to the cupin superfamily which is defined on the basis of a characteristic β-barrel tertiary structure. Despite significant deviations in thiol-substrates and amino acid sequence homology, several conserved features can be identified among TDO enzymes. A sequence alignment for selected TDO enzymes is provided in Supplemental information, Fig. S1. From this analysis, the 3-His active site motif appears to be a common feature among TDO enzymes. Beyond the active site 3-His residues, several conserved residues are observed in CDO enzymes across phylogenic domains (Y58, R60, H155, and Y157). For example, Y157 and H155 residues are present in both eukaryotic and prokaryotic enzyme forms. Moreover, both Y157F and H155A CDO variants exhibit abolished (or minimal) enzymatic activity [20,21]. Recent spectroscopic experiments on the catalytically inactive ferric enzyme suggest that these residues are involved in key outer-sphere interactions with the substrate-bound active site to facilitate catalysis. By contrast, the C93 involved in C93-Y157 cross-link formation is only observed in mammalian CDO enzymes. In bacterial enzymes this residue is replaced by a glycine suggesting that this cross-link is not catalytically essential. In support of this hypothesis, C93A and C93S variants of mammalian CDO retain catalytic activity, albeit with decreased k_{cat} [13,20]. X-ray crystallography and computational models suggest that R60 is involved in charge stabilizing of the L-Cys carboxylate group. This Arg residue is absent in mammalian and bacterial ADO enzymes which catalyze the O_2-dependent oxidation of 2-aminoethanethiol (cysteamine). It has also been noted that Ser153, His155 and Tyr157 may form a "*catalytic triad*' similar to those observed among hydrolase or transferase enzymes [22].



Scheme 1. CDO catalyzed oxidation of L-cysteine to produce cysteine sulfinic acid.

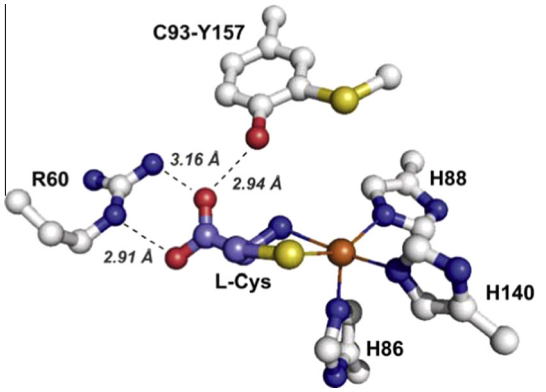


Fig. 1. 1.60 Å resolution X-ray crystal structure for the substrate-bound CDO active site at pH 8.0 (pdb code 4IEV) [10].

This seems unlikely as S153 is not universally conserved across phylogenic domains similar to H155 and Y157. No experiments to data provide any insight into the catalytic role of these residues.

Previous studies suggest that mammalian CDO exhibits high substrate and stereoselectivity [23–26]. For example, it has been reported that L-homocysteine is an inhibitor of CDO but its potential as a substrate has not been carefully evaluated [27]. In fact detailed characterization of any non-natural substrates is severely underreported. In this work, a variety of commercially available thiol-substrates were utilized to evaluate the substrate-specificity of wild-type CDO cloned from *Mus musculus*. Substrates were selected to evaluate specific active site interactions and cross-reactivity among other physiological TDO substrates [ADO (*cysteamine*), MPDO (*3-mercaptopropionate*), and MSDO (*mercapto-succinate*)]. The steady-state kinetic parameters and coupling efficiencies for O_2-consumption and sulfinic acid formation are reported. In all substrates evaluated, NMR spectroscopy, differential $^{16}O/^{18}O$-incorperation, and tandem LC–MS/MS were employed to confirm formation of the appropriate sulfinic acid product.

Materials and methods

Enzyme purification

Recombinant mouse CDO was expressed in *Escherichia coli* BL21(DE3) pLysS competent cells (Novagen) and purified using a 10 L bioreactor (New Brunswick Scientific Bioflo100) as previously described [14]. The as-isolated CDO enzyme typically contains ~50% (±10%) of the C93-Y157 cross-link as observed by SDS PAGE. Therefore, prior to use, isolated CDO is converted to the fully modified enzyme as described elsewhere [13]. All preparations were assayed for ferrous and ferric iron content spectrophotometrically as previously described [15,28]. Typical ferrous iron incorporation within purified CDO is ~ 70% (±10%). For clarity, the concentrations reported in enzymatic assays reflect the concentration of ferrous iron within samples of CDO (Fe^{II}-CDO).

NMR kinetic study

NMR kinetic studies were performed on a 300 JEOL nuclear magnetic resonance spectrometer (Pleasanton, CA). All measurements were made in Wilmad NMR tubes (standard wall, 5 mm O.D., precision, 507-PP-7). For each reaction, fully modified CDO (typically 2–25 μM) was added to a buffered substrate solution in D_2O (sodium phosphate buffer, 50 mM NaCl, pD 7.5) to initiate the reaction at ambient temperature (20 ± 2 °C). Reaction points were terminated by heat shock at 95 °C for 2 min followed by spin-filtration to remove denatured protein. Final concentration